

Studies on nucleotide levels and electron transport genes of *Clostridium acetobutylicum* P262

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**Dedicated to my Grandmothers,
Helen Caputo Tribuiani and
Maria Josephine Malatesta Santangelo**

CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "General regulations for the degree of Ph.D." I as supervisor of the candidate Joseph D. Santangelo, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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"I don't know what you mean by 'glory,'" Alice said.
Humpty Dumpty smiled contemptuously. "Of course you don't—till I tell you!. I meant 'there's a nice knock-down argument for you!'"
"But 'glory' doesn't mean 'a nice knock-down argument,'" Alice objected.
"When *I* use a word," Humpty Dumpty said, in rather a scornful tone, "it means just what I choose it to mean—neither more nor less."

Humpty Dumpty to Alice,
In: *Through the Looking Glass*,
by Lewis Carroll.

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Abstract

Clostridium acetobutylicum P262 is an endospore-forming Gram positive anaerobic bacterium, and for many years this organism has been used in the industrial fermentation for the production of acetone and butanol from carbohydrate substrates. The aims of this thesis included studies on small phosphorylated molecules involved in energy metabolism and cell differentiation, and an investigation into the genetics and molecular biology of *C. acetobutylicum* electron transport genes.

To facilitate quantitation of nucleoside triphosphates in extracts of *C. acetobutylicum*, a chromatographic data acquisition and analysis system was constructed. Samples were prepared from *C. acetobutylicum* cultures by treatment with formic acid, and nucleotides contained in these extracts were separated by strong anion exchange HPLC. The developed manual integration system features the ability to collect and store chromatographic data, allowing for multiple integration using different calibration curves.

Nucleoside triphosphate profiles were obtained from batch fermentations of the *C. acetobutylicum* P262 wild type, sporulation deficient (*spo-1*) and solvent deficient (*cls-1*) strains. The nucleoside triphosphate profiles of the wild type and *spo-1* mutant were similar and were characterized by a trough in nucleotide levels which occurred just prior to the pH break point, the onset of the stationary growth phase, clostridial stage formation and the transition from the acidogenic to the solventogenic phase. The nucleoside triphosphate concentrations during the exponential growth phase were much lower than those found during the stationary phase. Exponential phase nucleotide levels in the *cls-1* mutant were comparable to those observed in the wild type and *spo-1* mutant. Unlike the wild type and *spo-1* strains, the *cls-1* mutant, which does not switch to solventogenesis, did not demonstrate an increase in nucleotide levels after the cessation of cell division. The involvement of nucleotide levels, particularly that

of GTP, in the differentiation of *C. acetobutylicum* was indicated by the effect of inhibitors, which have been shown to decrease ribonucleotide levels in other organisms and cause an increase in sporulation.

The antibacterial agent metronidazole, was used as a tool for the isolation of *C. acetobutylicum* electron transport genes. Since it was desired to clone these genes in *Escherichia coli*, and investigation into the activation of metronidazole by *E. coli* strains was necessary. *E. coli* strains with lesions in their DNA repair systems were more susceptible to metronidazole than wild type strains. However, it has been reported that DNA repair deficient strains of *E. coli* that also had a diminished ability to reduce chlorates and nitrates were no more susceptible to metronidazole than their wild type parents (Jackson et al., 1984; Yeung et al., 1984). To isolate a suitable *E. coli* cloning host for the selection of *C. acetobutylicum* electron transport genes which activated metronidazole, transposon mutagenesis of the *recA* *E. coli* strain CC118 with *TnphoA*, was used to construct the *recA*, metronidazole resistant *E. coli* strain F19. F19 was shown to have diminished nitroreductase activity, which was presumed to be responsible for the metronidazole resistant phenotype. However, the *recA* mutation renders *E. coli* F19 highly susceptible to the reduced toxic intermediates of metronidazole.

The *E. coli* F19 *recA*, nitroreductase deficient mutant was used for the isolation of *C. acetobutylicum* genes on recombinant plasmids which activated metronidazole. Twenty five *E. coli* F19 clones which contained different recombinant plasmids were isolated. The clones were tested for nitroreductase, pyruvate-Fd-oxidoreductase and hydrogenase activities. Nitroreductase and pyruvate-Fd-oxidoreductase activity was not demonstrated in any of the isolated clones, and only one clone tested positive for hydrogenase activity.

DNA hybridization and restriction endonuclease mapping revealed that four of the *C. acetobutylicum* insert DNA fragments on recombinant plasmids were linked in an 11.1 kb chromosomal fragment. It was determined that this 11.1 kb

fragment contained at least two regions responsible for activating metronidazole. The one gene responsible for making *E. coli* F19 extremely sensitive to metronidazole was localized to a 2 kb region. The nucleotide sequence of this 2 kb region was determined and two truncated open reading frames and one complete open reading were present. The complete open reading frame was shown to be responsible for activating metronidazole. The deduced amino acid sequence of this open reading frame was determined to be 160 amino acids in length, and database searches showed good similarity to flavodoxin proteins from many organisms. Based on alignments to the amino acid sequences of these flavodoxins, as well as the fact that Chen and Blanchard (1979) reported that reduced flavodoxin can transfer electrons to metronidazole, the sequence corresponding to this *C. acetobutylicum* metronidazole activating gene was identified as coding for a flavodoxin gene.

The role of flavodoxin in *C. acetobutylicum* and other organisms is presented. Possible relationships between the cloned *C. acetobutylicum* flavodoxin gene and metronidazole sensitivity are discussed.

Abbreviations

A	adenosine
A ₂₅₄	absorbance at 254 nm
aa(s)	amino acid(s)
ABE	acetone-butanol-ethanol
Ap	ampicillin
ATCC	American Type culture collection
ATP	adenosine 5'-triphosphate
bp(s)	base pair(s)
C	cytidine
CAMM	<i>Clostridium acetobutylicum</i> minimal medium
CBM	<i>Clostridium</i> basal medium
cfu	colony forming units
Cm	chloramphenicol
CoA	coenzyme A
CsCl	cesium chloride
CTP	cytosine 5'-triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
Em	erythromycin
ECAM	<i>E. coli</i> anaerobic medium
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
Fd	ferredoxin
G	guanosine
GTP	guanosine 5'-triphosphate
h	hour(s)
kb	kilobase pairs
Km	kanamycin
LB	Luria-Bertani broth
min	minute(s)
mRNA	messenger RNA
M _r	relative molecular mass
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nt	nucleotides
NTP	nucleotide triphosphate
OD ₆₀₀	optical density at 600 nm
ORF	open reading frame
ori	origin of replication
ox	oxidized

p	plasmid
PAGE	polyacrylamide gel electrophoresis
Pho	alkaline phosphatase
<i>phoA</i>	gene coding for alkaline phosphatase
Pollk	Klenow fragment of <i>E. coli</i> DNA polymerase I
P _R	rightward promoter (λ)
r	(superscript) resistance
red	reduced
RBS	ribosome binding site
RNA	ribonucleic acid
s	second(s)
s	(superscript) sensitivity
SDS	sodium dodecyl sulfate
sp(p)	specie(s)
T	thymidine
TAE	tris-acetate EDTA buffer
Tc	tetracycline
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
U	units of enzyme activity
UV	ultraviolet (light)
UTP	uridine 5'-triphosphate
v/v	volume/volume
w/v	weight/volume
XGal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
XP	5-bromo-4-chloro-3-indolyl phosphate
::	novel joint (fusion)
[]	plasmid carrier state
Δ	deletion

Chapter 1

General introduction and literature review

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Chapter 1

General introduction and literature review

In many countries, the development of chemical industries based on abundant supplies of inexpensive fossil fuels has led to the relinquishment of traditional fermentation routes employed for chemical production. In the consumer oriented western world, the economies of many nations are reliant on the abundance of inexpensive fossil fuels. Since the advent of the oil shortage in the early 1970's, there has been fresh interest in the production of chemicals by the fermentation of renewable resources, and the acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* has often been included (Spivey, 1978).

1.0 History of the Acetone-Butanol-Ethanol fermentation

The history and development of the ABE fermentation was recently review by Jones and Woods (1986a), and this review provided the basis for the majority of the history presented below.

A shortage of natural rubber in the early 1900's led to investigations on the feasibility of producing synthetic rubber. Butyl or isoamyl alcohol could be used as substrates for the production of butadiene or isoprene respectively (Killeffer, 1927; Gabriel, 1928). Chaim Weizmann was a chemist working at Manchester University, who chose to research the preparation of synthetic rubber. Not long after the start of his work, Weizmann realized that such a process could not succeed without an abundant supply of butanol (Hastings, 1978).

The production of butanol by microbial fermentation was first reported by Pasteur in 1861, whereas the production of acetone by microbial fermentation was only reported later (Schardinger, 1905). Although Chaim Weizmann was not a bacteriologist, he investigated the feasibility of butanol production via a

microbial route for use in the manufacture of synthetic rubber. Between 1912 and 1914, Weizmann isolated an organism which he called BY (Gabriel, 1928), later to be named *C. acetobutylicum*, which was able to breakdown a variety of starchy substrates and produce good yields of acetone and butanol (McCutchan and Hickey, 1954).

Concurrent with Weizmann's investigations, the British firm of Strange and Graham Ltd. filed an English patent application for the production of acetone and butanol by microbial fermentation. This process was based on an organism isolated in 1911 by Fernbach, that had the ability to ferment potatoes, but not maize starch, to produce butanol (Gabriel, 1928; Gabriel and Crawford, 1930). In 1914, Strange and Graham Ltd. built a plant in King's Lynn, for the production of butanol from potatoes (Gabriel, 1928).

The start of World War I in August of 1914, gave impetus to further develop the ABE fermentation. Acetone, aside from being a general solvent, was required for the manufacture of cordite which is used in small arms munitions (Killeffer, 1927). Necessary quantities of acetone were not available to the British army, and so the British War Office contracted Strange and Graham Ltd. to produce acetone at their King's Lynn plant (Gabriel and Crawford, 1930). The process of manufacturing acetone from potatoes using the Fernbach strain was inefficient and hence production did not meet the requirements of the British army (Gabriel, 1928).

In his laboratory at Manchester University, Weizmann was very successful at producing quantities of butanol sufficient for his research (Hastings, 1978). The British government requested all scientists to undertake work that would contribute to the war effort. Although Weizmann considered acetone to be a by-product of the fermentation, he soon realized its importance and quickly brought his work to the attention of the proper authorities (Hastings, 1978). In due course, an official research team was assembled to work under Weizmann's guidance, and all supplies and materials were provided by the British government for the production of acetone from maize using the Weizmann

process (Weizmann, 1963; Walton and Martin, 1979). Pilot scale fermentation plants were developed at a number of sites in southern England (see Jones and Woods, 1986a), and eventually a full scale production facility was built at the Royal Naval Cordite factory where six distilleries were adapted for the production of acetone from maize using the Weizmann process (Gabriel, 1928). The Strange and Graham Ltd. plant at King's Lynn was also converted to the Weizmann process, resulting in increased production of acetone (Ross, 1961).

In 1916, the British government could not afford to supply cereal grains used for the production of acetone via fermentation (Hastings, 1978). The fermentation was therefore transferred to Canada, and was operated under the guidance of Herbert Speakman with Weizmann remaining in London as the principal adviser (Gabriel, 1928; Weizmann, 1963; Hastings, 1978). The year 1917 saw the entry of the United States of America into the war, and ABE fermentation plants were initiated at two distilleries located in Terre Haute, Indiana (Ross, 1961). The cessation of hostilities in November of 1918, brought with it a decreased demand for acetone which resulted in the closure of these North American fermentation facilities.

The Allied War Board auctioned the two distilleries in Terre Haute, and they were purchased in 1919 by a consortium of American businessmen operating under the name of the Commercial Solvents Corporation of Maryland (Gabriel, 1928). At this time the E.I. DuPont de Nemours Co. of Wilmington Delaware was developing quick drying nitrocellulose lacquers for use in the rapidly recovering automobile industry (Walton and Martin, 1979). Butanol and its ester, butyl-acetate were necessary solvents used in the production of these nitrocellulose lacquers. Butanol production was re-initiated in 1920 at the Terre Haute plants which were now operating under the control of the Commercial Solvents Corporation. The Commercial Solvents Corporation obtained the Majestic Distillery of Peoria Illinois in 1923, where further expansion of the ABE fermentation took place (Gabriel, 1928). By the end of 1927 there were 96 fermentors in operation at the Peoria plant with an additional 52 fermentors at the Terre Haute plants.

Economics eventually led to a joint venture between the Commercial Solvents Corporation and the Distiller's Co. of Great Britain where a fermentation facility was built in Brombrough England based on molasses as the fermentation feedstock (Gibbs, 1983). Under the guidance of J. Hastings, the ABE fermentation became operational at this site in 1935 using a phage immunized strain of *C. saccharoacetobutylicum* (Gibbs, 1983). Due to the success of this fermentation facility, the relatively inexpensive substrate, molasses, became the feedstock of choice for the ABE fermentation.

The worldwide patent for the Weizmann process, in which the Commercial Solvents Corporation had sole licence, expired in 1936 (McCutchan and Hickey, 1954). In the United States, the production of acetone and butanol from molasses expanded, with companies other than the Commercial Solvents Corporation building fermentation plants in the port cities of Philadelphia Pennsylvania and Baltimore Maryland where molasses could be obtained via waterways from sugar cane growing regions. Fermentation facilities for the production of solvents were also built in other countries including Australia, Brazil, Egypt, Formosa (Taiwan), India, Japan and South Africa (Rose, 1961; Ross, 1961; Abou-Zeid et al., 1978; Spivey, 1978).

The Second World War brought with it another increase in the demand for acetone to be used in the manufacture of munitions (Hastings, 1971). Production of solvent containing fermentation liquor exceeded the capacity of the batch distillation processes employed for harvesting the solvents, and successful efforts were made to use multiple column continuous distillations facilities which were built for the industrial alcohol plants (Hastings, 1978). Difficulties in the importation of molasses during the Second World War, led to the demise of this fermentation in Great Britain (Hastings, 1971). The demand for acetone used in the war effort was therefore met by the production facilities in North America, which were operating at capacity (Wynkoop, 1943).

The demise of this fermentation in the western world began in the late 1940's, when the importance of propylene and ethylene, which were by-products of the

cracking of petroleum, was realized by the petrochemical industry (Gibbs, 1983). Although the production of acetone and butanol by fermentation continued through the 1950's, the contribution made by fermentation to the total output diminished rapidly (Rose, 1961). Breakthroughs in catalysis enabled excellent yields of a wide variety of industrially important chemicals from petroleum, which were much less expensive than those derived from fermentation (Gibbs, 1983). The incorporation of molasses into cattle feed caused an escalation in the price of this substrate, hence providing the final devastating blow to this fermentation (Hastings, 1978).

Acetone and butanol were also produced via fermentation in eastern block countries including China, Czechoslovakia and the USSR (Dyr et al., 1958; Nakhmanovich and Shcheblykina, 1959; Yarvenko, 1964; Lukina, et al., 1972; Herrero, 1983). Information concerning these production facilities is however scattered and incomplete.

In a few countries, a combination of political, economic and climatic factors enabled the survival of some ABE fermentation plants. China currently produces 50% of its acetone requirements using this fermentation process (J.S. Chiao, personal communication). In South Africa an economically viable process for the production of acetone and butanol from molasses operated from 1937 to 1981 (Jones and Woods, 1986a). This plant was reopened for a short period in 1982, however technical problems along with the quality, cost and availability of molasses resulted in closure of this fermentation facility (Jones and Woods, 1986a).

1.1 Overview of the ABE fermentation

The bacterium, *C. acetobutylicum*, is a strictly anaerobic, Gram positive spore forming rod (O'Brien and Morris, 1971), and microscopic observations of actively growing cells reveal motile, straight rods with rounded ends, occurring singly and in pairs (Spivey, 1978). This microorganism can be isolated from many natural sources, including soil, corn and potatoes (Volesky et al., 1981).

Carbohydrates from a wide variety of sources are readily fermented to acetic and butyric acid intermediates, which are converted to the neutral end products, acetone, butanol and ethanol. The morphological (Jones et al., 1982), physiological (Prescott and Dunn, 1959; Walton and Martin, 1979) and biochemical (Duong et al., 1983; Zeikus, 1983) changes which occur during the course of this fermentation have been well documented (Jones and Woods, 1986a; Rogers, 1986).

The conventional batch ABE fermentation carried out by *C. acetobutylicum* features two distinct physiological stages (Jones et al., 1982). The initial acidogenic growth phase is characterized by highly motile phase dark rod shaped cells which are rapidly dividing. During this logarithmic phase of growth, sugars are converted to acetic and butyric acids, resulting in a decrease in the available carbohydrate nutrient supply and a drop in the pH of the medium (Fig. 1.1). The relative amounts of acetic and butyric acids present during this growth phase have been shown to be related to the ratio of acetone and butanol produced during the second or solventogenic growth phase (Martin et al., 1983).

A switch in the carbon flow from the acid producing pathways to the solvent producing pathways is correlated with the onset of the solventogenic growth phase. At this point, the acid concentration in the medium reaches a maximum which corresponds to the "pH breakpoint" (Jones et al., 1982). This pH breakpoint was identified early on as being crucial for solventogenesis to occur (Speakman, 1920; Davies and Stephenson, 1941). During the solventogenic stage, cell division ceases and the reassimilation of organic acids begins. Continued uptake and consumption of carbohydrate occurs, resulting in the production of the neutral solvents acetone, butanol and ethanol.

Granulose is a high molecular weight storage polyglucan containing mainly 1-4 linked D-glucopyranose units (Reysenbach et al., 1986). Just prior to the pH breakpoint, there is an intracellular accumulation of granulose and a slowing of cell motility. The cellular morphology associated with the solventogenic phase is

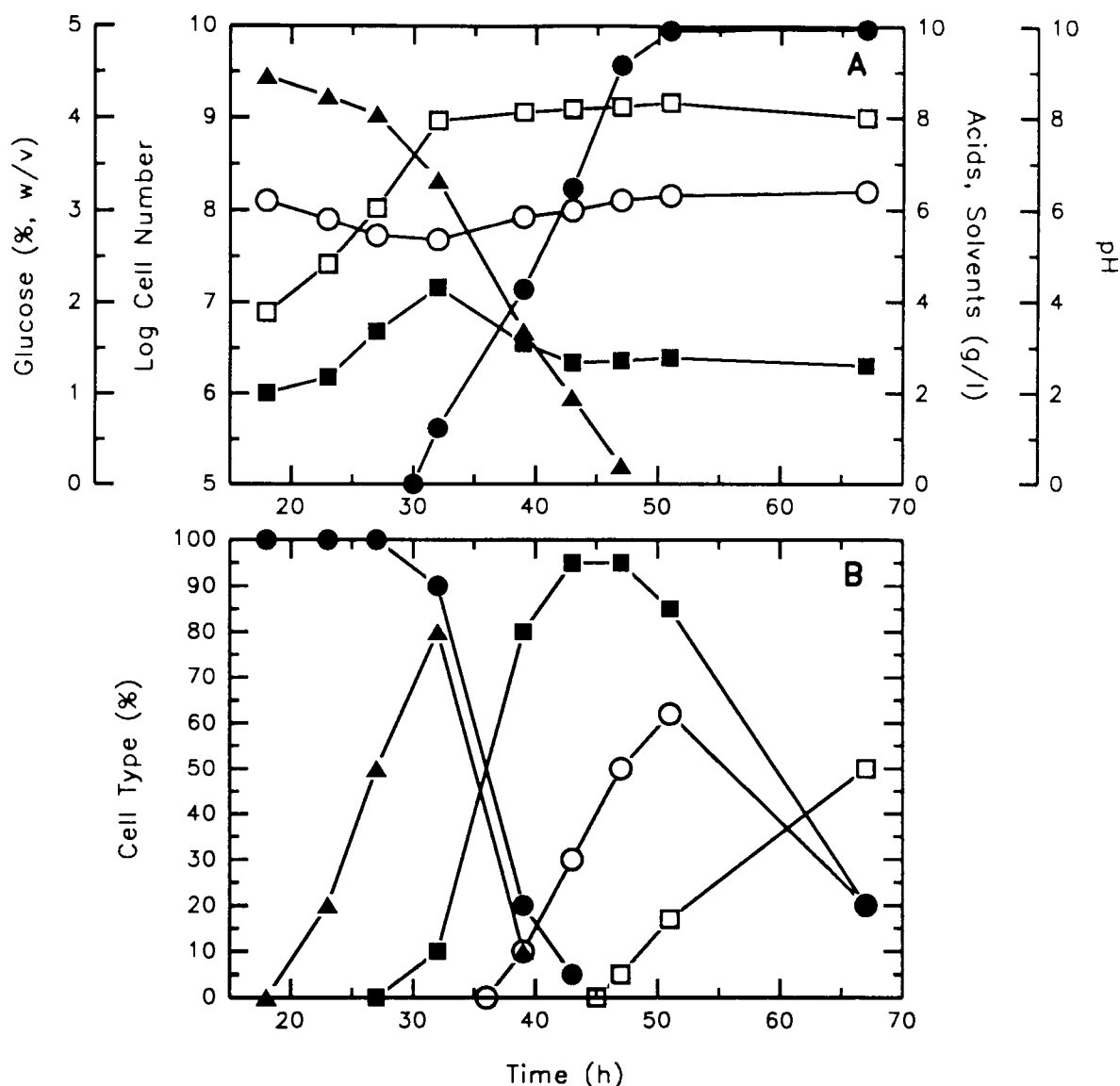


Figure 1.1 Physiological (A) and morphological (B) profiles for a typical *C. acetobutylicum* batch fermentation. Symbols for graph (A) correspond to the following: ▲, glucose concentration (% w/v); ■, total acids (g l⁻¹); ●, total solvents (g l⁻¹); □, log₁₀ cell number; ○, pH. Symbols for graph (B) correspond to the following: ▲, motility; ■, phase bright clostridial cells; ●, phase dark rods; ○, forespore containing cells; □, mature spores. All data in plot (B) are expressed as a percentage of the total cell count. (Data from Santangelo et al., 1989).

characterized by phase bright, encapsulated, granulose filled, non-motile cells. These cells have a swollen rod, cigar shaped morphology, and are commonly referred to as clostridial forms (Jones et al., 1982; Long et al., 1984a; Reysenbach et al., 1986). Spivey (1978) reported that by monitoring these morphological changes associated with the stages of the ABE fermentation, an experienced observer can assess the progress of the fermentation to within a few hours.

A typical industrial ABE fermentation is carried out with a relatively low starting concentration of fermentable sugars (approximately 6.0 to 6.5% w/v) (Spivey, 1978; McNeil and Kristiansen, 1985). Solvent toxicity, particularly that of butanol, begins at a concentration of approximately 2% (w/v) (van der Westhuizen et al., 1982). Since one third of the available carbohydrates are converted to solvents, the use of sugar levels greater than 6.0 to 6.5% (w/v) is futile. A typical batch ABE fermentation yields 15 to 22 g l⁻¹ of solvents with an acetone:butanol:ethanol ratio of 3:6:1 (Spivey, 1978; Jones et al., 1982).

Industrial batch fermentations were generally stopped prior to sporulation, when the concentration of solvents had reached maximum levels. The loss of spore forming capability during continuous culture has been associated with the loss of ability to make solvents (Gottschal and Morris, 1981a). Similar results were obtained by Long et al. (1983, 1984b) who studied the relationship between sporulation and solvent production in batch culture. However, reports concerning sporulation in industrial batch fermentations show that only a small proportion of the cells proceed to produce mature spores (Jones et al., 1982). In fermentations where high solvent levels were reached, clostridial forms did not develop towards sporulation, but degenerated into non-viable cells.

1.2 Biochemistry and physiology of *C. acetobutylicum*

The conversion of carbohydrates to acids, gases and neutral solvents by *C. acetobutylicum* has been extensively recorded (Doelle, 1975; Gottschalk, 1979; Hartmanis and Gatenbeck, 1984). Reviews by Rogers (1986) and Jones and Woods (1986a) have outlined the general relationship between the biochemical pathways and the fermentation profiles of the clostridia, whereas a review by Häggström (1985) has focused primarily on energy metabolism.

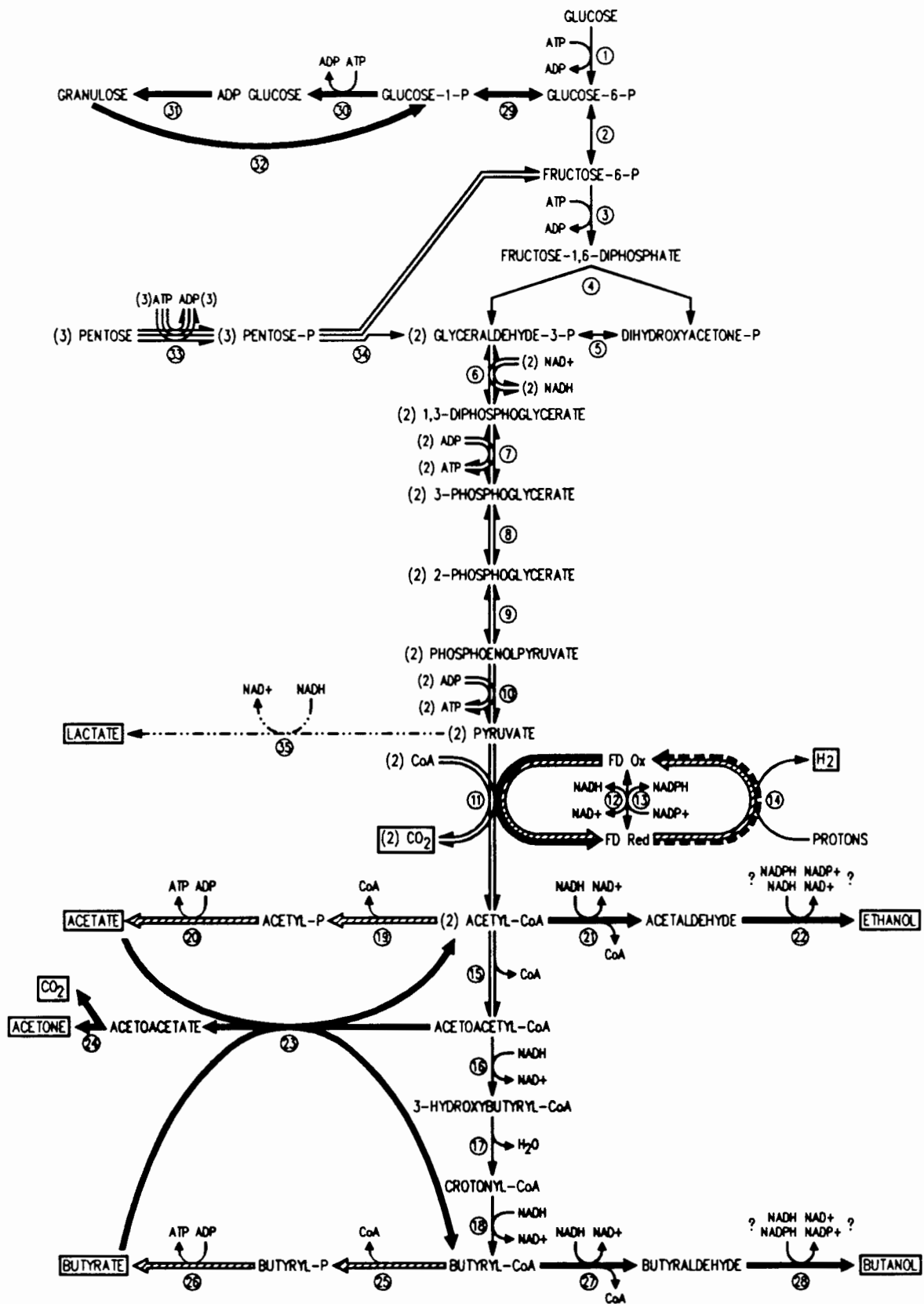
1.2.1 Central pathways

In *C. acetobutylicum*, the breakdown of sugars to pyruvate proceeds via the same pathways in both the acidogenic and solventogenic stages of growth (Fig. 1.2). The catabolism of hexose sugars to pyruvate proceeds via the glycolytic pathway

where 1 mol of hexose yields 2 mol of pyruvate with the net production of 2 mol of ATP and 2 mol of NADH. *C. acetobutylicum*, is able to metabolize pentose sugars via the pentose phosphate pathway (Zeikus, 1980; Volesky and Szczesny, 1983). Three mol of pentose are phosphorylated to pentose 5-phosphate at the expense of 3 mol of ATP. Three mol of pentose 5-phosphate are converted to 2 mol of fructose 6-phosphate plus 1 mol of glyceraldehyde 3-phosphate by the action of phosphopentose isomerase, phosphopentose epimerase, transketolase and transaldolase enzymes. Fructose 6-phosphate and glyceraldehyde 3-phosphate are intermediates of the glycolytic pathway which can be further metabolized to pyruvate. Hence the breakdown of 3 mol of pentose yields 5 mol of pyruvate with the net production of 5 mol of ATP and 5 mol of NADH (Fig. 1.2).

The phosphoroclastic breakdown of pyruvate is accomplished by the enzyme pyruvate ferredoxin (Fd) oxidoreductase in the presence of coenzyme A (CoA) and oxidized Fd to yield carbon dioxide, acetyl-CoA and reduced Fd. Acetyl-CoA is one of three key intermediates for the branched fermentation pathways leading to both acid and solvent production. Acetyl-CoA acetyltransferase is the enzyme responsible for the formation of another key intermediate, acetoacetyl-CoA, which is formed in a single step by the condensation of 2 molecules of acetyl-CoA. Butyryl-CoA, the third key intermediate, is formed from acetoacetyl-CoA by the action of 3 enzymes: 3-hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase. Both 3-hydroxybutyryl-CoA dehydrogenase and butyryl-CoA dehydrogenase consume reducing power in the form of NADH. The presence of these 5 enzymes responsible for the breakdown of pyruvate to butyryl-CoA has been demonstrated in *C. acetobutylicum* (Waterson et al., 1972; Hartmanis and Gatenbeck, 1984; Wiesenborn et al., 1988). The fate of these 3 intermediates differs during the acidogenic and solventogenic growth stages as discussed below.

Figure 1.2 (Facing page) Biochemical pathways in *C. acetobutylicum*. Reactions which predominate during the acidogenic phase are represented by arrows with thick slashed lines, whereas reactions which predominate during the solventogenic phase are represented by arrows with thick solid lines. Reactions common to both pathways are represented by arrows with thin lines. The thick dashed solid line for the oxidation of reduced Fd represents a reduction in enzyme activity during the solventogenic growth phase. The thin dash-dot-dot line for the conversion of pyruvate to lactate represents an inducible pathway not normally operating in *C. acetobutylicum*. All end products of the fermentation (including gases) are enclosed in boxes. Enzymes are indicated by circled numbers as follows: (1) hexokinase; (2) phosphoglucose isomerase; (3) phosphofructokinase; (4) aldolase; (5) triose phosphate isomerase; (6) glyceraldehyde 3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) phosphoglyceromutase; (9) enolase; (10) pyruvate kinase; (11) pyruvate-Fd-oxidoreductase; (12) NADH-Fd-oxidoreductase; (13) NADPH-Fd-oxidoreductase; (14) hydrogenase; (15) thiolase (acetyl-CoA acetyl transferase); (16) 3-hydroxybutyryl-CoA dehydrogenase; (17) crotonase; (18) butyryl-CoA dehydrogenase; (19) phosphate acetyltransferase (phosphotransacetylase); (20) acetate kinase; (21) acetaldehyde dehydrogenase; (22) ethanol dehydrogenase; (23) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (24) acetoacetate decarboxylase; (25) phosphate butyltransferase (phosphotransbutyrylase); (26) butyrate kinase; (27) butyraldehyde dehydrogenase; (28) butanol dehydrogenase; (29) phosphoglucomutase; (30) ADP-glucose pyrophosphorylase; (31) granulose (glycogen) synthase; (32) granulose phosphorylase; (33) pentokinase; (34) a combination of phosphopentose isomerase, phosphopentose epimerase, transketolase and transaldolase; (35) lactate dehydrogenase.



1.2.2 Acid producing pathways

Acetyl-CoA and butyryl-CoA are the two key intermediates in the production of acetic and butyric acids. The respective formation of acetate and butyrate from acetyl-CoA and butyryl-CoA occurs via an analogous set of reactions. However, the enzymes involved in these reactions have been shown to be unique to each branched pathway (Andersch et al., 1983; Hartmanis and Gatenbeck, 1984; Jones and Woods, 1986a; Rogers, 1986). The CoA containing intermediates are converted to their corresponding acyl-phosphates, acetyl phosphate and butyryl phosphate, by the action of phosphate acetyltransferase and phosphate butyryltransferase respectively. These acyl-phosphates are metabolized to acetate and butyrate by the action of acetate kinase and butyrate kinase with the generation of one ATP for each acyl-phosphate converted. A CoA-transferase has been shown to be responsible to butyrate formation in *C. kluyveri* (Stadtman, 1952) and *C. aminobutyricum* (Hardman and Stadtman, 1963), however no such transferase involved in butyrate synthesis has been detected in either *C. acetobutylicum* or *C. beijerinckii* (Andersch et al., 1983).

Apart from the production of acetic and butyric acids, *C. acetobutylicum* can also convert pyruvate to lactate under certain conditions (Jones and Woods, 1986a). The production of lactic acid does not occur under ideal conditions. This pathway operates as a less efficient alternative to the acetate and butyrate producing pathways, to allow for energy generation and the oxidation of NADH to continue when the mechanism for proton and electron disposal by the generation of hydrogen gas is inhibited (Eliasberger, 1930; Simon, 1947; Kim et al., 1984; Kim and Zeikus, 1985). Although not an acid, acetoin has also been shown to accumulate when electron disposal is blocked (Doremus et al., 1985). While the biosynthetic pathway for the production of acetoin in *C. acetobutylicum* remains to be elucidated, it has been suggested that this compound could be produced as an intermediate under conditions in which the lactate pathway becomes operational (Doremus et al., 1985). Acetoin production is critical in that it is a structural analog of acetoacetate which can reduce the efficiency of acetone production.

1.2.3 Solvent producing pathways

While acetic and butyric acids are produced from the intermediates acetyl-CoA and butyryl-CoA, a switch in the carbon flow directs these same two intermediates towards the production of ethanol and butanol respectively (Fig. 1.2). Like the production of acetate and butyrate, the production of ethanol and butanol proceeds via a set of analogous reactions. Acetyl-CoA and butyryl-CoA are converted to their respective aldehydes, acetaldehyde and butyraldehyde, via a dehydrogenation step. It is not known whether two unique and independent aldehyde dehydrogenases exist, although this seems likely as ethanol production has been shown to occur independent of acetone and butanol production (Jones and Woods, 1986a; Bertram et al., 1990). Ethanol production may not be regulated by the same mechanisms as butanol production, and it is possible that ethanol is produced constitutively during both acidogenesis and solventogenesis. At least two separate alcohol dehydrogenases are responsible for the metabolism of acetaldehyde and butyraldehyde to ethanol and butanol respectively. It has been demonstrated in both *C. acetobutylicum* and *C. beijerinckii*, that butanol production occurs via a NADPH dependent butanol dehydrogenase (George and Chen, 1983; Rogers, 1986), whereas ethanol production appeared to be carried out via the action of a NADH dependent ethanol dehydrogenase. However, Welch et al. (1989) have recently purified and characterized a NADH dependent butanol dehydrogenase from *C. acetobutylicum*, indicating that multiple alcohol dehydrogenases may exist in this organism, and hence may function independently under various growth conditions. While it has been assumed for some time that a specific NADPH dependent alcohol dehydrogenase was responsible for the production of butanol, the isolation of solvent minus mutants (Bertram et al., 1990) indicates that an NADH specific enzyme is involved (Grupe and Gottschalk, 1990).

The production of acetone from the third key intermediate, acetoacetyl-CoA, is a two step process where the enzyme acetoacetate decarboxylase carries out the irreversible conversion of acetoacetate to acetone with the concurrent production of carbon dioxide (Andersch et al., 1983; Ballongue et al., 1985). Acetoacetate is

provided by the acetoacetyl-CoA:acetate/butyrate:CoA transferase which acts upon acetoacetyl-CoA (Hartmanis et al., 1984; Wiesenborn et al., 1989a; Cary et al., 1990).

Acetic and butyric acids are reassimilated during the solventogenic phase, resulting in an increase in the pH of the medium. This reassimilation only occurs with an accompanying consumption of sugars (Davies and Stephenson, 1941; Hartmanis et al., 1984). Early studies showed that the addition of extraneous acetate and butyrate enhanced the yields of acetone and butanol (Reilly et al., 1920; Bernhauer and Kurschner, 1935). A later study by Wood et al. (1945), showed that when ^{14}C labeled acetate was added to solvent producing cultures of *C. acetobutylicum*, 55% of the label was recovered in butanol and 15% of the label was recovered in acetone. Similarly, the addition of ^{14}C labeled butyrate resulted in 85% and 2% of the label being recovered in butanol and acetone respectively. More recent studies also employing ^{14}C labeled butyrate showed almost identical results. In these experiments, the half life of butyrate in the medium was determined to be approximately 20 min and a complete turnover of acids occurred within 2 h (P. Rogers, personal communication). Therefore, the majority of the acetate and butyrate taken up by *C. acetobutylicum* is rapidly converted to butanol. The effect of short chain fatty acids on the fermentation yields of *C. acetobutylicum*, has continued to be the focus of more recent studies (Gottschal and Morris, 1981b; Bahl et al., 1982a; Martin et al., 1983; Clark et al., 1989; Wiesenborn et al., 1989a; Husemann and Papoutsakis, 1990).

Although a number of different mechanisms have been postulated for the uptake of acetic and butyric acids (see Jones and Woods, 1986a), recent results indicate that this reassimilation is directly coupled to the production of acetone by way of acetoacetyl-CoA:acetate/butyrate:CoA transferase. A study by Andersch et al. (1983) clearly showed that acetate and butyrate can both act as a CoA acceptor during the conversion of acetoacetyl-CoA to acetoacetate resulting in the conservation of the CoA unit by the simultaneous production of acetyl-CoA or butyryl-CoA. The decarboxylation of acetoacetate by the acetoacetate

decarboxylase has been postulated to pull the transferase reaction toward the formation of acetoacetate (Hartmanis et al., 1984).

1.2.4 Energy and electron distribution

Ferredoxin (Fd) is a major electron carrier in the metabolic pathways of *C. acetobutylicum*. It is a small acidic iron-sulfur containing protein that is capable of accepting and donating electrons at a very low potential (Jones and Woods, 1986a). In *C. acetobutylicum* 4 enzymes are known to require Fd for complete functionality. As mentioned above, the pyruvate-Fd-oxidoreductase transfers electrons from the phosphoroclastic breakdown of pyruvate to oxidized Fd to yield reduced Fd. The hydrogenase enzyme in turn utilizes the electrons carried by reduced Fd and together with protons, forms molecular hydrogen. The enzyme NADPH-Fd-oxidoreductase can also utilize reduced Fd in the controlled production of NADPH. This may be the only route for the production of NADPH, which is required for biosynthesis, since most clostridia appear to lack the enzymes required for the oxidation of glucose 6-phosphate that produce NADPH (Jungermann et al., 1973). The analogous yet separate enzyme, NADH-Fd-oxidoreductase, cycles electrons between Fd and NADH, and hence is key to the electron distribution in this organism (Jungermann et al., 1973; Petitdemange et al., 1976).

The growth of anaerobes is limited by the rate of energy producing reactions (Thauer et al., 1977). In *C. acetobutylicum* the glycolytic breakdown of 1 mol glucose to 2 mol pyruvate yields a net production of 2 mol ATP and 2 mol of NADH. During acidogenesis, 1 mol of ATP is produced per mole of acyl phosphate converted to acid end product. Therefore, the net energy yield obtained when 1 mol of glucose is converted to 2 mol of acetate is 4 mol of ATP. Since 2 mol of acetyl-CoA are required for the production of 1 mol of butyryl-CoA, the net energy yield obtained when 1 mol of glucose is converted to 1 mol of butyrate is 3 mol of ATP. In a normal batch fermentation, the acetate to butyrate ratio is approximately 2:3 (mol:mol) (Jones and Woods, 1986a). Accounting for the acetate to butyrate ratio, Rogers (1986) calculated the ATP yield for the

acidogenic growth phase to be about 3.25 mol ATP per mol of glucose consumed, with a thermodynamic efficiency of approximately 62%. Apart from ATP produced during glycolysis, no further ATP production occurs during solventogenesis via the synthesis of acetone, butanol and ethanol. Therefore, the ATP balance for the solventogenic growth phase drops to 2 mol ATP per mol of glucose consumed.

The energetically more favourable production of acetate results in a net generation of 2 mol NADH. During both acidogenesis and solventogenesis 2 mol of reducing power, in the form of NADH, are consumed in the conversion of 2 mol of acetyl-CoA to 1 mol of butyryl-CoA. Therefore, even though butyrate production yields less ATP than does acetate production, it is redox neutral. Since the breakdown of glucose to acetyl-CoA yields 2 mol of NADH, and considering that only a portion of the resulting acetyl-CoA is converted to butyrate, excess NADH must either accumulate, or be cycled through Fd via the NADH-Fd-oxidoreductase. The latter would result in reduced Fd and hence require increased hydrogen production for electron disposal and recycling of Fd to its oxidized state. The solvent producing pathways, but not the acid producing pathways, provide additional routes for the disposal of NADH, where both aldehyde dehydrogenases, as well as the alcohol dehydrogenases, each consume 1 mol of NADH per mol of substrate converted to product. This indicates that any accumulation of NADH and hence the activities of the NADH-Fd-oxidoreductase and hydrogenase enzymes, all play a role in the switching of the carbon flow from acid production to solvent production.

1.3 Triggers and regulation in *C. acetobutylicum*

Both the induction of solventogenesis and the induction of sporulation appear to be linked to the inhibition of vegetative growth and normal cell division (Jones and Woods, 1986a). The development of chemically defined media that support the complete life cycle of *C. acetobutylicum* (Andersch et al., 1982; Long et al., 1983; Monot and Engasser, 1983) has greatly facilitated studies involved in the elucidation of the external triggers that influence differentiation to solvent

production and to sporulation. Furthermore, the development of mutagenic systems and the isolation of *C. acetobutylicum* mutants (Jones et al., 1982; Long et al., 1984a; Bowring and Morris, 1985; Jones and Woods, 1986b) has also aided this area of research. However, the mechanisms by which these signals are interpreted to molecular events which adjust specific enzyme activities are only just starting to be realized.

1.3.1 Regulation of solvent production

The external catalysts that trigger the metabolic shift from acid production to solvent production have been extensively studied. A number of significant changes in the fermentation pattern are associated with the end of the initial phase of growth. A decrease in the pH of the medium, which is related to an increase in the concentration of acid end products, cessation of cell division and a decrease in the rate of hydrogen production all characterize the onset of the solventogenic growth phase.

The role of pH has long been recognized as being important to the outcome of the ABE fermentation. Almost all studies concerning the ABE fermentation mention the importance of a "pH breakpoint" in allowing the fermentation to proceed to solventogenesis. Studies have shown that cultures which are maintained at a high pH produce mainly acids, whereas cultures maintained at a low pH produce solvents (Jones and Woods, 1986a; Rogers, 1986). The pH optima for solvent production does however vary from strain to strain (see Jones and Woods, 1986a). While attainment of a low pH is necessary for solvent production, it has been shown that this low pH is not in itself a sufficient trigger for solvent production (Gottschal and Morris, 1981b; Long et al., 1984a).

The fall in the pH of the medium is a consequence of the production of acetate and butyrate. These organic acids are toxic, and in their undissociated form they are able to partition in the cell membrane and behave as uncouplers which permit the entry of protons into the cell (Kell et al., 1981; Husemann and Papoutsakis, 1986). A recent study showed that a transition from a neutral pH to an acidic pH resulted in the formation of transmembrane lipids that increase the

rigidity of the cell membrane which in turn alters the bioelectrochemical processes that occur across this membrane (G. Zeikus, personal communication). At sub-critical concentrations, the accumulation of acid end products results in a decrease in the specific growth rate of the cell, although substrate utilization and cell metabolism continue (Herrero, 1983). It has been suggested that the switch to solvent production is a detoxification mechanism which allows the cell to avoid the inhibitory effects that would occur if acid end products were allowed to reach critical levels (Jones and Woods, 1986a).

The pH of the cell environment determines the percentage of acids which are in their undissociated form. Undissociated butyric acid in particular, was shown to be essential in the regulation of solvent production (Bahl et al., 1982a). If the critical level of undissociated butyric acid was not obtained because of either too high a pH or too low an initial substrate concentration, solvent production did not occur (Bahl et al., 1982a; Bahl and Gottschalk, 1985).

Monot et al. (1984) showed that blocking the function of the membrane bound ATPases results in a decrease in the internal pH of the cell. By decreasing the internal pH, the concentration of undissociated acids is increased. Under these conditions, there was a reduction in the maximum biomass levels with an enhancement of solvent production. Unlike most other organisms, bacteria that produce weak acids are not able to maintain a constant internal pH (Riebeling et al., 1975; Baronofsky et al., 1984). In these organisms, the proton translocating ATPase is used to control the internal pH in response to fluctuations in the external pH. By altering the internal pH at the expense of ATP, *C. acetobutylicum* is able to maintain a more or less constant pH gradient across its membrane as the pH of the external medium decreases (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang et al., 1985; Andreesen et al., 1989). The shift to solvent production and the reassimilation of acids may therefore be a necessary event so that the cell is able to maintain this pH gradient across its membrane. If the concentration of undissociated acids was allowed to rise, the cost to the cell in terms of ATP, would eventually be greater than the amount of ATP produced by the generation of these acids.

Solvent production only occurs with accompanying glucose consumption. Studies of fermentations where the carbon source was limited showed that solvent production did not occur and hence only acids were produced (Gottschal and Morris, 1981*a*; Bahl et al., 1982*a*; Fond et al., 1984; Long et al., 1984*a*). In these studies, the concentration of acid end products did not reach the threshold concentration required for the initiation of solvent production. Attempts to produce solvents under conditions of nitrogen limitation in the presence of excess glucose, yielded similar results. Using batch cultures, Long et al. (1984*a*) showed that low concentrations of ammonia (9.0 mM) resulted in the consumption of less than one-third of the available glucose and no solvent production. Studies using ammonium limited chemostat cultures also showed that solvent production did not occur (Gottschal and Morris, 1981*a*; Andersch et al., 1982). Apparently the threshold level of acids was not reached and therefore no induction of solventogenesis occurred. Limiting the concentration of phosphate or sulfate in batch and continuous cultures displayed more success at inducing solvent production than did glucose or ammonium limitation (Bahl et al., 1982*b*; Bahl and Gottschalk, 1985). High yields of acetone and butanol were obtained in a phosphate limited two-stage continuous culture, and apparently this process was uninterrupted for 1 year without a change in cell activity (Bahl et al., 1982*b*).

1.3.2 Regulation of electron flow

In the solvent producing clostridia, the pathways for acid production alone do not provide for the disposal of excess NADH produced during glycolysis. These clostridia have the ability to produce hydrogen and therefore can process both excess electrons and protons through this route. During acidogenesis, a major portion of the electron flow is directed to hydrogen production, whereas the carbon flow is directed to acid production. Recent studies have shown that during this growth phase more hydrogen is produced than is theoretically possible from the phosphoroclastic breakdown of pyruvate alone (Martin et al., 1982; Kim and Zeikus, 1985).

The 2 enzymes NADH-Fd-oxidoreductase and hydrogenase play a pivotal role in controlling the direction of electron flow, as well as the acetate:butyrate ratio (Jones and Woods, 1986a). Hydrogenase activity is reduced when the partial pressure of hydrogen is high, and studies with *C. butyricum* showed that an increase in the partial pressure of hydrogen resulted in an increase in the amount of butyrate produced with respect to acetate (Crabbendam et al., 1985). An increase in the carbon flow through the glycolytic pathway results in an increase in the level of acetyl-CoA, and this increase has been shown to stimulate the activity of NADH-Fd-oxidoreductase in the clostridia (Jungermann et al., 1976). Therefore, an efficient route for the disposal of excess NADH is provided, allowing for the more energy favourable production of acetate.

In *C. acetobutylicum* the shift from acidogenesis to solventogenesis is accompanied by a decrease in hydrogen production and an increase in CO₂ production. Less hydrogen is produced than would be expected from the oxidation of pyruvate, indicating that during this growth phase both carbon and electron flow are primarily directed to solvent production. This switch in carbon flow appears to be directly linked to the reduction of hydrogen production, and Kim and Zeikus (1985) observed that the specific rate of hydrogen production decreased in stages during the course of a batch fermentation. As expected, the highest rate of hydrogen production occurred during the initial growth phase, which could be correlated with a high rate of glucose consumption. A decrease in the metabolic activity resulted in a decrease in hydrogen production, however specific hydrogenase activity associated with the whole cell did not alter. It was concluded that this decrease in hydrogen production was due to a decline in the availability of reduced Fd resulting from a decrease in glucose consumption. These authors noted a second, more significant, decrease in the rate of hydrogen production which correlated with the shift from acid production to solvent production. In this case the specific hydrogenase activity with respect to the whole cell did decrease.

The specific hydrogenase activity in whole cells from acid producing cultures was shown to be approximately 2.2 times higher than that measured from

solvent producing cultures (Kim and Zeikus, 1985). Kim and Zeikus (1985) demonstrated that hydrogenase activity was not affected by either pH or acid concentration, and concluded that a decrease in hydrogen production during solventogenesis is a result of regulation of hydrogenase production as opposed to an inhibition of enzyme activity. In contrast to these findings, Andersch et al. (1983) reported that hydrogenase activity was similar in both acid and solvent producing cells. However, hydrogenase activity in solvent producing cells could only be detected in the assay after an initial lag period of 10 to 15 min. These authors concluded that the hydrogenase from the solvent producing cells was present in an inactive form and the conditions used for the assay activated the enzyme after a lag period.

Hydrogenase activity, and therefore solvent production, could be altered by changing the partial pressure of hydrogen in the culture vessel. It was shown that by using hydrogen to increase the headspace pressure to between 100 and 250 kPa, the yields of butanol and ethanol could be increased (Gapes et al., 1982). Increasing the partial pressure of hydrogen in the headspace resulted in a decrease in hydrogenase activity. A similar study was carried out by Yerushalmi et al. (1985), where the headspace of the fermentation vessel was pressurized with either hydrogen or helium. When hydrogen was used to pressurize the fermenter to between 270 and 1 500 kPa, butanol and ethanol yields were increased by an average of 18 and 13% respectively. A much lower increase in butanol and ethanol production was noted when helium was used to pressurize the culture vessel. A similar, yet weaker effect was noted when *C. acetobutylicum* cultures were not agitated, allowing for the partial pressure of hydrogen to rise naturally (Doremus et al., 1985; Yerushalmi and Volesky, 1985). Together, these results verify that when conditions are reached where the partial pressure of hydrogen is increased, there is a resulting decrease in hydrogen production and a stimulation of butanol and ethanol production, but not acetone production. Under these conditions, the H^+/H_2 redox potential is lowered and the flow of electrons from reduced Fd to molecular hydrogen is thermodynamically unfavourable (Jungermann et al., 1971b). Because the hydrogenase was

inhibited, the flow of electrons from reduced Fd was shifted to the generation of NADH and NADPH via the actions of the respective Fd-oxidoreductase. Stimulation of ethanol and butanol production was therefore necessary for the disposal of electrons carried by these reduced pyridine nucleotides.

Carbon monoxide (CO) is known to reversibly inhibit the function of hydrogenases by reacting with iron present in the active site of the enzyme (Thauer et al., 1974; Mortenson and Chen, 1975). Sparging cultures of *C. acetobutylicum* with CO has been shown to alter the fermentation balance, resulting in an increase in the butanol yield (Kim et al., 1984; Datta and Zeikus, 1985; Meyer et al., 1985). Acetate and butyrate uptake can also be enhanced when cultures are sparged with CO. Increasing the partial pressure of the culture vessel with CO to between 100 and 150 kPa resulted in a 50% decrease in hydrogen production, a 50 to 200% increase in butyrate uptake and a 10 to 15% increase in butanol yield (Datta and Zeikus, 1985). It is also important to note that CO has been shown to inhibit the acetoacetate decarboxylase enzyme which is responsible for the formation of acetone. Therefore, the ratio of solvents can be controlled by a combination of CO sparging and the addition of organic acids (Datta and Zeikus, 1985).

The fate of reduced Fd determines the electron distribution in the cell in that it can either transfer electrons via hydrogenases to produce hydrogen, or electrons can be transferred via the appropriate Fd-oxidoreductase to yield reduced pyridine nucleotides. Therefore, the activities of NADH-Fd-oxidoreductase, NADPH-Fd-oxidoreductase and hydrogenase in effect control the direction of carbon flow by controlling the electron flow within the cell (Jungermann et al., 1973, 1976; Jones and Woods, 1986a). Glyceraldehyde 3-phosphate dehydrogenase and NADH-Fd-oxidoreductase both function in controlling the levels of NAD⁺ and NADH in the cell. Furthermore, CoA and acetyl-CoA are known to act as allosteric inhibitors and activators of NADH-Fd-oxidoreductase respectively (Jungermann et al., 1973). Since a high concentration of NADH inhibits the production of NADH via the NADH-Fd-oxidoreductase, and considering the role of CoA and acetyl-CoA in controlling this enzyme, the ratios

of acetyl-CoA:CoA and $\text{NAD}^+:\text{NADH}$ must play a principal role in the regulation of electron flow, and hence carbon flow, in *C. acetobutylicum* (Datta and Zeikus, 1985; Jones and Woods, 1986a). In addition, it has been postulated that the ratios of these compounds can function as sensors for both ATP regeneration and hydrogen production (Datta and Zeikus, 1985).

1.3.3 Sporulation and stationary phase events

Bacillus subtilis has been the organism of choice for studying the metabolic and genetic events associated with endospore formation in bacteria. From these studies the process of endospore formation has been simplified as occurring in two discrete phases: (i) the initiation phase where growing cells recognize and respond to changes in the environment where rapid growth is no longer supported, and (ii) the differentiation phase where genes whose products are required for sporulation are expressed in a defined and genetically determined order (Sonenshein, 1985). It has been extensively demonstrated that in *B. subtilis* spore formation can be initiated by exposing rapidly multiplying cells in a liquid medium to a nutritionally poor medium (Sterlini and Mandelstam, 1969; Losick et al., 1986; Sonenshein, 1989). Unfortunately, similar nutrient shift down procedures do not induce sporulation in the clostridia, and it has been shown that these organisms sporulate only under conditions where growth is limited in the presence of an exogenous carbon and energy source (Murrell, 1967; Hickey and Johnson, 1981; Woods and Jones, 1986). In *C. acetobutylicum* in particular, neither glucose nor ammonium limitation succeeded in the induction of solventogenesis or sporulation (Long et al., 1983, 1984a). In fact, these studies showed that at intermediate concentrations of glucose or ammonia, the yield of endospores was proportional to the substrate concentration.

It appears that in *C. acetobutylicum*, sporulation is initiated by the same factors which induce solvent formation, which include a decrease in pH and the accumulation of acid end products. This suggests that a parallel switch for both solventogenesis and sporulation exists. As mentioned previously, the findings of Gottschal and Morris (1981a) that correlated a loss of spore forming ability with a

loss of the ability to produce solvents, support this idea of a parallel switch. However, the isolation of asporogenous mutants that maintain the ability to produce solvents, demonstrates that initiation of spore formation is not a prerequisite for solvent production. Asporogenous *C. acetobutylicum* mutants blocked before (SPO 0) or after (SPO II-VI) forespore septum initiation, retained the ability to produce solvents (Jones et al., 1982; Long et al., 1984b).

Gottschal and Morris (1981a) and Jones et al. (1982) both reported the isolation of a second class of asporogenous *C. acetobutylicum* mutants. Apart from being asporogenous, these isolates, termed *cls* mutants (Jones et al., 1982), did not develop into clostridial forms and therefore lacked all the stationary phase characteristics associated with solventogenesis. Solvent production, capsule production, granulose production and endospore formation were all absent in these *cls* mutants (Long et al., 1984a, 1984b). The *cls* mutants derived from *C. acetobutylicum* P262, were isolated after treatment with ethyl methane sulfonate (EMS). EMS is a direct mutagenic agent known to cause point mutations by alkylating purine bases (Birge, 1981). Assuming that the *cls* mutants were derived from single point mutations, the observation that normal wild type characteristics are restored by reversion of such mutations suggests that the induction of the stationary phase events is linked by some common regulatory mechanism. However, it is not known if the *cls* mutation is a result of a defect in coordinate induction of the events associated with solventogenesis or if it is related to an inability to attain or maintain a particular physiological state necessary for the onset of solventogenesis (Jones and Woods, 1986a).

Apart from the solvent producing asporogenous mutants mentioned above, mutants lacking other individual stationary phase events have been isolated (Jones and Woods, 1988). These include mutants that are unable to produce granulose or extracellular capsule material, as well as mutants that produce altered levels of solvents (Long et al., 1984b; Dürre et al., 1986; Reysenbach et al., 1986; Rogers, 1986). The isolation of these mutants demonstrates that although the induction of stationary phase events may be linked, the individual pathways leading to these events function independently. While solvent production,

granulose accumulation and capsule formation do not appear to be sporulation specific events (Long et al., 1984b), the initiation of these events may be a prerequisite for initiation of sporulation. Definitive experimental support for this hypothesis is limited, however it is interesting to note that a single mutagenic treatment has never been reported to lead to the isolation of a *C. acetobutylicum* mutant that lacks all stationary phase events except sporulation.

1.4 Genetic studies of *C. acetobutylicum*

The development of systems for the transfer of nucleic acids into *C. acetobutylicum* has lagged behind the ability to study genes from this bacterium in other organisms such as *E. coli* and *B. subtilis*. High levels of extracellular and cell bound deoxyribonuclease (DNase) activity are associated with the majority of *C. acetobutylicum* strains (see Jones and Woods, 1986b). This has hampered attempts at transferring DNA into *C. acetobutylicum* via many methods such as protoplast fusion and transfection. Recent advances in the construction of suitable cloning vectors and transformation techniques for *C. acetobutylicum*, have provided the much needed basis for future genetic manipulations of this organism. The rapid progress in these areas has been the subject of recent publications (Jones and Woods, 1986b; Jones and Woods, 1988; Young et al., 1989a, 1989b; Minton et al., 1990a, 1990b).

1.4.1 Cloned *C. acetobutylicum* genes

Apart from chemical mutagenesis, the initial approach used for genetic studies of *C. acetobutylicum*, was to clone and study clostridial genes in well characterized bacteria such as *E. coli* (Jones and Woods, 1986b).

The first genes cloned from *C. acetobutylicum* included an endoglucanase, a xylanase and some amino acid utilization genes (Efsthathiou and Truffant, 1986; Zappe et al., 1986, 1987, 1988). These genes appeared to be expressed from their own promoters, thereby demonstrating that *C. acetobutylicum* genes could function in *E. coli*. The importance of nitrogen metabolism in the life cycle of *C. acetobutylicum* led to the cloning of a glutamine synthetase gene (Usdin et al.,

1986). This *glnA* gene was also expressed from its own promoter and was regulated by nitrogen levels in *E. coli* (Janssen et al., 1988, 1990). Other *C. acetobutylicum* genes that have been cloned into *E. coli* include a lactate dehydrogenase (Contag et al., 1990), an α -amylase (J. van der Leyden, personal communication) and the endonuclease *Cac824I* (E.T. Papoutsakis, personal communication).

Attainment of a molecular level understanding of acidogenic and solventogenic pathway gene regulation has focussed most efforts towards cloning of these genes. Genes coding for the central pathway enzymes thiolase (G.N. Bennett, personal communication) and 3-hydroxybutyryl CoA dehydrogenase (Youngleson et al., 1989a) have been cloned and studied in *E. coli*. Initial reports (Youngleson et al., 1989a, 1989b) indicated that the 3-hydroxybutyryl CoA dehydrogenase formed part of a *but* operon which included an alcohol dehydrogenase. However, recent experiments reveal that while these two genes are linked on the *C. acetobutylicum* chromosome, they may function independently. (D.R. Woods, personal communication).

Acidogenic pathway genes coding for the two enzymes, butyrate kinase and phosphotransbutyrylase which are responsible for the conversion of butyryl-CoA to butyrate, have been cloned and were also found to be linked on a contiguous stretch of *C. acetobutylicum* chromosomal DNA (Cary et al., 1988; Wiesenborn et al., 1989b).

A NADPH dependent alcohol dehydrogenase was the first solvent pathway gene to be cloned (Youngleson et al., 1988, 1989b). Recently a NADH dependent butanol dehydrogenase has been cloned (G.N. Bennett, personal communication), indicating that more than one enzyme may be able to carry out the conversion of butyraldehyde to butanol. Other cloned solvent pathway genes include the acetoacetate decarboxylase (Gerischer and Dürre, 1990; Petersen and Bennett, 1990) and the acetoacetyl-CoA:acetate/butyrate:CoA transferase (Cary et al., 1990). Both of these genes were cloned in two independent laboratories, with each group isolating these genes from unique *C.*

acetobutylicum strains. Both groups showed that these two genes were linked on a contiguous piece of *C. acetobutylicum* chromosomal DNA (G.N. Bennett and P. Dürre, personal communications).

1.4.2 Genetic manipulation of *C. acetobutylicum*

The development of efficient gene transfer systems for *C. acetobutylicum* is essential for future genetic manipulations of this organism. Protoplast fusion and regeneration using *C. acetobutylicum* strain P262 (Allcock et al., 1982; Jones et al., 1985) and *C. saccharoperbutylacetonicum* strain N1-4080 (Reysset et al., 1987, 1988) has been reported. The efficiency of these systems was however low, most likely due to the high nuclease and autolysin activities associated with these organisms (Jones and Woods, 1986b). An autolysin deficient mutant (N1-4081) of strain N1-4080 was isolated, and proved to be more easily regenerated (Reysset et al., 1988).

Conjugal transfer of DNA into *C. acetobutylicum* was first reported by Reysset and Sebald (1985), who showed that the streptococcal plasmid pAM β 1 could be transferred to *C. acetobutylicum* strains 903 and ATCC 824 at a frequency approaching 10^{-6} per donor. The plasmid pAM β 1 was also shown to be conjugatively transferred to *C. acetobutylicum* strain NCIB 8052 using either *Streptococcus lactis* or *B. subtilis* as the donor (Oultram and Young, 1985; Oultram et al., 1987). Several other broad host range plasmids such as pIP501, pJH4, and pVA797 have been transferred to *C. acetobutylicum* via conjugation (Young et al., 1989a, 1989b). Furthermore, plasmids pVA797 and pAT187 have been shown to mobilise non conjugative plasmids such as pAM610 into *C. acetobutylicum* strains P262 (Yu and Pearce, 1986) and NCIB 8052 (Young et al., 1989a, 1989b) respectively. Recently, Williams et al. (1990) developed a conjugative transfer system using *E. coli* as the donor and *C. acetobutylicum* NCIB 8052 as the recipient. However, these authors reported that they were unable to successfully apply this system to other *C. acetobutylicum* strains such as DSM 1731, ATCC 824 and P262. Since *E. coli* has generally been utilized as the standard organism for genetic manipulations, this conjugation system is very attractive.

Transposons have been extensively employed in genetic studies of many organisms. In *E. coli* transposons have been used to identify promoters, to test for export mechanisms, to clone genes and to generate specific and polar mutations (de Bruijn and Lupski, 1984). Conjugative transposons are able to function in a wide variety of Gram positive hosts (Clewell and Gawron-Burke, 1986) including *C. acetobutylicum* (Young et al., 1989a, 1989b). Yu and Pearce (1986) reported the successful expression of the streptococcal transposon Tn917 in *C. acetobutylicum*, however these authors did not demonstrate whether the transposon was incorporated into the host chromosome, or remained on the plasmid used for transfer. Woolley et al. (1989) demonstrated that the conjugative transposons Tn916 and Tn1545 could be transferred from *Enterococcus faecalis* to *C. acetobutylicum* NCIB 8052. Both of these transposons harbour a tetracycline resistance (Tc^R) marker, and while Tn1545 was able to insert into the *C. acetobutylicum* chromosome at multiple sites, Tn916 had favoured sites of insertion. Bertram and Dürre (1989) showed that the conjugative transposons Tn925, Tn916 and Tn925:Tn917 could also be efficiently transferred from *E. faecalis* to the chromosome of *C. acetobutylicum*. Exemplifying the differences amongst *C. acetobutylicum* strains, these authors demonstrated that Tn916 could insert at multiple sites in the chromosome of strains ATCC 824, DSM 792 and DSM 1731.

Using the Tn916 conjugation/mutagenesis system, Bertram et al. (1990) isolated three types of mutants of *C. acetobutylicum* DSM 792, by selecting for colonies that were resistant to either allyl alcohol or 2-bromobutyrate. The type I mutant lacked the ability to produce acetone and butanol, type II produced reduced levels of solvents and type III showed an increase in butanol production. These mutants were asporogenous and lacked the ability to accumulate granulose. These latter characteristics could however be due to an effect of tetracycline (transposon marker), in an analogous fashion to the effect of chloramphenicol which inhibits sporulation in *B. subtilis*. These authors showed that the type I mutant contained only one copy of Tn916, and considering that both acetone and butanol production was absent, the hypothesis that there is global regulation for

the initiation of solventogenesis is strongly supported. The ability of the type I mutant to produce ethanol suggests that ethanol production may be independent from the induced solvent pathways for acetone and butanol production.

Subculturing the above transconjugants 15 times in the absence of selective pressure (Tc) for the transposon, resulted in approximately 50% of the colonies becoming sensitive to tetracycline while maintaining the mutant phenotype. Hybridization studies revealed that these Tc^s revertants did not contain the Tn916 transposon. This suggests that transposon excision in *C. acetobutylicum* is not precise, which is in contrast to results obtained with Tn916 in *E. coli* (Gawron-Burke and Clewell, 1984).

As an alternative to conjugation, lytic and lysogenic phages known to infect *C. acetobutylicum* could be developed into possible cloning vectors. Bacteriophages have been identified as the cause of abnormal fermentations (McCoy et al., 1944), and the initial characterization of some of these phages has been carried out (Hongo et al., 1968; Ogata and Hongo, 1979; Nieves et al., 1981). Although whole cell transduction of *C. acetobutylicum* has not been reported, the successful development of lysogenic phages such as $\phi 105$ as cloning vectors for *B. subtilis* (Errington, 1984) indicates that an analogous system could be developed for *C. acetobutylicum*. Further efforts to characterize and develop *C. acetobutylicum* phages are currently in progress (D.T. Jones, personal communication).

A procedure by which a high voltage electric discharge through a cell suspension facilitates the introduction of DNA into the cells, offers yet another alternative to conjugation. This procedure, known as electroporation, is believed to induce transient pores in the cell membrane through which exogenous DNA can enter the cell (reviewed by Fiedler and Wirth, 1988; Wirth et al., 1989). Successful transformation via electroporation has been reported for a number of Gram positive bacteria including members of the genera *Streptomyces*, *Streptococcus*, *Lactobacillus* and *Bacillus* (see Lucansky et al., 1988). Recently, Oultram et al. (1988) reported that *C. acetobutylicum* NCIB 8052 could be transformed by electroporation at frequencies approaching 3×10^3 transformants per μg DNA.

The convenience of transforming naked DNA and the ease of use of this procedure, may render it the method of choice for the introduction of foreign DNA into *C. acetobutylicum*.

1.4.3 Cloning vectors for *C. acetobutylicum*

Concurrent with developments concerning the introduction of DNA into *C. acetobutylicum* was the construction of suitable cloning vectors for use in this organism. While the occurrence of plasmids in the clostridia has been noted, none of these plasmids contain selectable markers suitable for genetic manipulations (Minton and Thompson, 1990). This has necessitated the *in vitro* construction of vectors by combining replicons and antibiotic resistance genes known to function in *C. acetobutylicum* (Truffaut et al., 1989; Minton et al., 1990a, 1990b; Yoshino et al., 1990).

As a starting point, Minton and co-workers cloned the antibiotic resistance genes for Erythromycin (Em), Tetracycline (Tc) and Chloramphenicol (Cm) into the pUC like cloning vectors pMTL20 and pMTL21 (Chambers et al., 1988) to yield pMTL20/21E, pMTL20/21T and pMTL20/21C respectively. The next step was to include replicons known to function in the clostridia. Four different sources of clostridial replicons were cloned into these vectors, and provided resultant plasmids that were able to function in *C. acetobutylicum* (Minton et al., 1990a). Interestingly, none of these replicons supported efficient plasmid replication in *B. subtilis*, indicating that clostridial replicons are functionally distinct from those found in aerobic Gram positive bacteria (Gruss and Ehrlich, 1989).

Features such as a broad host range and structural stability, led these workers to base further *C. acetobutylicum* vector construction on the streptococcal plasmid pAM β 1, and therefore a detailed study of the replication region of this plasmid was performed (Swinfield et al., 1990). As a result of this analysis, a 2.9 kb *EcoRI-HpaI* fragment carrying the replication region of pAM β 1 was cloned into the plasmid pMTL20E resulting in plasmid pMTL500E (Swinfield et al., 1990; Minton et al., 1990b). Plasmid pMTL500E retains all of the desirable features of the pUC like cloning vector pMTL20. These features include a multiple cloning

site polylinker which resides within the *lacZ'* region, the *bla* gene for ampicillin selection in *E. coli* and an *E. coli* origin of replication. Due to the broad host range of pAM β 1, the vector pMTL500E is also able to replicate in *B. subtilis* as well as *C. acetobutylicum*. Analogous procedures led to the construction of plasmids pMTL500C and pMTL500T (from pMTL20C and pMTL20T) where the Gram positive antibiotic resistance markers were chloramphenicol and tetracycline respectively.

Segregational instability in the absence of selective pressure, has been shown to be a problem with vectors constructed for use in *C. acetobutylicum* (Truffaut et al., 1989; Yoshino et al., 1990). In the absence of Em, pMTL500E also showed segregational instability in both *B. subtilis* and *C. acetobutylicum* (Minton et al., 1990a). Janni re et al. (1989) showed that certain pAM β 1 derived cloning vectors for use in *B. subtilis* also showed segregational instability. However, one of these *B. subtilis* vectors, pHV1431, contained an additional 2.12 kb of pAM β 1 DNA and was much more stable in the absence of selective pressure. Insertion of this 2.12 kb fragment into the multiple cloning site of pMTL500E (=pMTL531E) significantly improved the segregational stability in both *B. subtilis* and *C. acetobutylicum* (Minton et al., 1990a). Molecular analysis of the 2.12 kb region revealed 2 open reading frames, one of which coded for a protein that showed good similarity to the resolvases of the Gram positive transposon Tn917 (Minton et al., 1990a). It has been suggested that the resolvase like proteins present on other Gram positive plasmids such as pI524 and pIP404, can mediate plasmid stability by maintaining the plasmid population in the monomeric state (Garnier et al., 1987; Rowland and Dyke, 1988). A similar role may be carried out by the resolvase like protein identified on pMTL531E (Minton et al., 1990a). Since the multiple cloning site of pMTL531E was disrupted by the resolvase containing DNA, the plasmid pMTL500E(*res*⁺) was constructed such that the multiple cloning site of pMTL500E remained intact (Minton et al., 1990a). This plasmid had a similar segregational stability to pMTL531E.

To further enhance the usefulness of the pMTL500 series of vectors, the *lac* promoter element of pMTL500E was replaced with the *C. pasteurianum* Fd

promoter element, such that the AUG start codon of Fd became that of the *lacZ'* gene. To allow for regulatory control, a synthetic *lac* operator was inserted between the -10 region and the ribosome binding site of this promoter element (at position +1) (Minton et al., 1990b). The resulting plasmid was designated pMTL500F, and it was shown that in *E. coli*, transcription from the Fd promoter could be regulated by a *lacI* repressor protein produced from a coexisting plasmid (Minton et al., 1990a). A similar control mechanism for pMTL500F in *C. acetobutylicum* is currently being developed (Minton et al., 1990a) analogous to a system that was developed for *B. subtilis* (Peschke et al., 1985).

1.5 Future prospects for the ABE fermentation

The lack of economic competitiveness of the ABE fermentation for the production of chemical feedstocks currently inhibits exploitation of this process. Knowledge gained with respect to the biochemistry, physiology, molecular biology and genetics of *C. acetobutylicum* has just about moved the prospects for this fermentation from being future to being current.

Marketing techniques in the western world have altered the perceptions of the consumer, such that the purchase of naturally derived products is desirable at all costs. With particular respect to the ABE process, there may be a small niche for products which contain solvents derived via fermentation. One example is fingernail polish remover, where the acetone content is low enough that the additional cost for the naturally derived solvent may not prohibitively increase the price of the final product.

The utilization of inexpensive alternative substrates such cellulosic waste, are of concern to many fermentations. However, application of future developments in this area to the ABE process will depend on the ability to genetically introduce these systems into *C. acetobutylicum*. Methods for the genetic manipulation of *C. acetobutylicum* have been successfully developed, and indeed Minton et al. (1990a, 1990b) have shown good expression of *C. thermocellum celA* and *celC* genes in *C. acetobutylicum*.

Complementing the genetic manipulation of *C. acetobutylicum* by introduction of foreign DNA, the development of conjugative transposon mutagenesis systems provides a powerful and convenient route for the isolation of *C. acetobutylicum* strains with desirable fermentation characteristics.

Although it was not discussed in this introduction, advances in the areas of process technology and downstream processing will no doubt have a marked affect on the re-emergence of the ABE fermentation (Gottschalk and Bahl, 1981).

Despite the current abundance of oil, it is a non-renewable resource. If nations truly want to eliminate oil as the basis for their economies, fundamental and applied research on the ABE fermentation must be welcomed and encouraged.

1.6 Overview of metronidazole

Metronidazole was used extensively in this study, thereby necessitating a brief discussion on this compound. This introduction is not intended to be a comprehensive review of the scientific literature, but rather provides enough information to allow for an understanding of why and how it was used as a research tool.

1.6.1 History and development of metronidazole

Similar to the history and development of the ABE fermentation, the history and development of metronidazole is colourful and a joy to discover.

Catteral (1976) wrote that in the 1950's, "at the majority of clinics there were queues of sad looking unhappy women...with wet and stained underwear and poor morale...nor was there any satisfactory treatment to offer them". The disease was known as trichomoniasis, and the causative agent had been shown to be *Trichomonas vaginalis*. Infection could persist for many years, making life a misery for the patient.

In 1954, the laboratories of Rhône-Poulenc in Paris, France instituted a research programme to search for a compound effective against *T. vaginalis* (McFadzean, 1986). Many compounds were tested, and in 1956, Despois et al. reported that a

crude extract from a streptomycete was active against the parasite. The active antibiotic was shown to be azomycin (Fig. 1.3) which had been discovered two years previously by Japanese workers (McFadzean, 1986). Following this lead, many derivatives of azomycin were synthesized and eventually in 1957, metronidazole was born (Fig. 1.3). Of all the compounds developed, metronidazole provided the best balance between activity and toxicity. Using mice infected with *T. vaginalis*, Cosar and Julou (1959) were the first to report the effectiveness of orally administered metronidazole. Clinical trials were instituted shortly thereafter (Durel et al., 1959), and metronidazole was introduced for prescription use in 1960 in France and the UK.

It is common for more active or better tolerated drugs to overtake their predecessors in the marketplace. This has not occurred with metronidazole (McFadzean, 1986) and after 30 years, it is still the drug of choice for the treatment of trichomoniasis.

This may have been the end of the story for metronidazole if it was not for the careful observations of certain people such as a dental surgeon named David Shinn. Shinn was a registrar at King's College Dental School in London, and in 1962 he had been treating a female patient with acute ulcerative gingivitis (McFadzean, 1986). The patient received the normal treatment of chromic acid and peroxide which was applied to the ulcers. On her next consultation, the patient's gingivitis was cured. Not believing that the miraculous results were due to his treatments, Shinn questioned this patient and discovered that she was receiving metronidazole treatment for trichomoniasis. Intrigued by his discovery, he treated six more ulcerative gingivitis cases with metronidazole, and all were rapidly cured. Shinn reported his results and encouraged others to test the effectiveness of metronidazole against acute ulcerative gingivitis (Shinn, 1962).

It had previously been established that large numbers of two organisms, *Borrelia vincenti* and *Bacteroides fusiformis*, were present in the ulcerative lesions of this gingivitis. In conjunction with May and Baker pharmaceuticals, who distributed

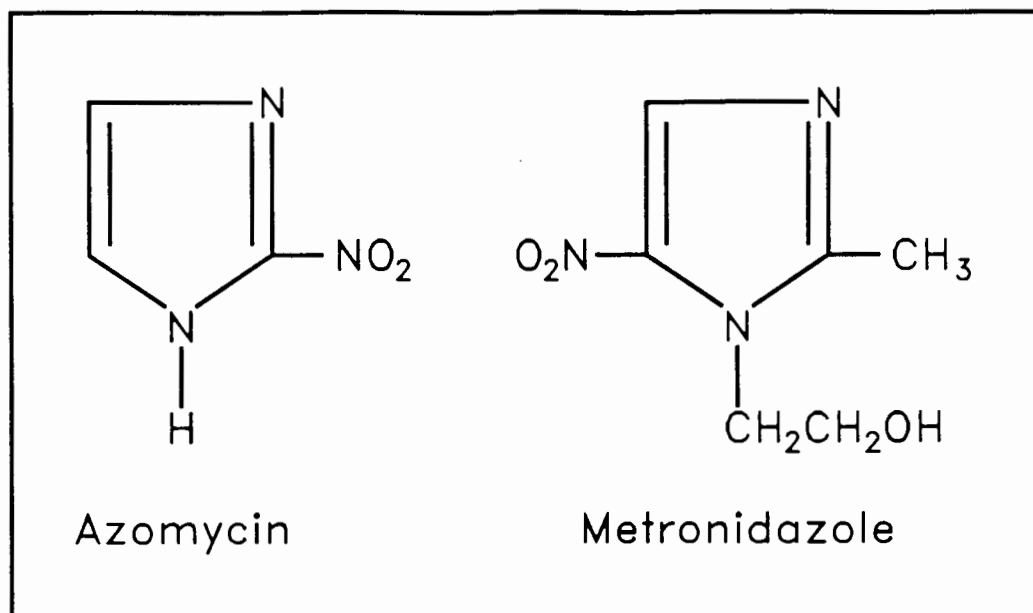


Figure 1.3 A comparative drawing showing the chemical composition of azomycin, and the chemical composition of its derivative, metronidazole.

metronidazole in the UK, Shinn was able to show *in vitro* killing of *B. fusiformis* by metronidazole. A confirmation of these results was published (Davies et al., 1964), and Shinn became one of the best known dentists in the world. Sponsored by May and Baker, Shinn visited dental schools in Canada and the USA with a view of moving there to practise dentistry. On his return to the UK, he announced his decision to remain in England as he was not prepared to give up the pleasure of watching Arsenal play football on a Saturday (McFadzean, 1986).

It was also shown that *in vitro*, metronidazole was active against *Entamoeba histolytica*, the causative agent of amoebic dysentery (Powell, et al., 1966). This amoeba, the trichomonad and the two bacteria associated with ulcerative gingivitis all had one thing in common; they grew anaerobically. May and Baker decided to test metronidazole against other anaerobic bacteria, particularly members of the genus *Clostridium*. Again, metronidazole was shown to have *in vitro* activity which was at least as effective as reference antibiotics used as controls (Freeman et al., 1968).

It had been shown that 98% of the viable bacteria of the large intestine were anaerobic, and were primarily members of the genus *Bacteroides* (Drasar et al.,

1966). Furthermore, clostridial infections were known to be a problem in causing gas gangrene shortly after limb amputation, and the source of the infection was believed to be from the patients own faeces (Parker, 1969). It was proposed to administer metronidazole to prevent this auto-infection, however this proposal did not gain acceptance for quite a number of years (McFadzean, 1986).

Advances were made in the ability to culture anaerobic bacteria, and so the realization of their importance in disease was becoming clear. However, this realization was only being recognized by small groups of people willing to challenge common medical opinion (McFadzean, 1986). Studies had shown that prophylactic metronidazole treatment was virtually 100% effective in controlling post operative sepsis by *Bacteroides* species following elective gynaecological surgery (Willis et al., 1974, 1975). These and other reports were viewed with great scepticism by the medical community, and even the highly regarded *New England Journal of Medicine* published a somewhat cynical report entitled "Beware - the anaerobe bandwagon" (Page, 1974). More and more results showed the efficacy of metronidazole in the treatment of post operative sepsis. Eventually institutions such as the Luton and Dunstable Hospital north of London, where many of the initial metronidazole studies were performed, concluded that it was unethical to withhold metronidazole prophylaxis when undertaking hysterectomy operations. In 1978, the Luton group stated that after 618 hysterectomies where metronidazole treatment was included, there had been no post operative anaerobic infections (McFadzean, 1986).

Similar results and conclusions were found when using prophylactic metronidazole treatment prior to and after other types of abdominal surgery. Metronidazole treatment was shown to be effective in controlling post operative sepsis after colonic surgery (Goldring et al., 1975; Willis et al., 1977), appendicectomy (Leigh et al., 1974; Willis et al., 1976) and obstetric surgery (Willis et al., 1978).

Since anaerobic bacteria are the principal agents behind sepsis following abdominal surgery, it is not surprising that metronidazole was found to be very

effective at treating other, non-surgically induced anaerobic infections. Metronidazole treatment has been effective against brain abscesses (Ingham et al., 1975, 1977), dental infections (Hood, 1978; Rood and Murgatroyd, 1979), bacterial meningitis (Berman et al., 1978), lung infections (Tally et al., 1975) and many other important and miscellaneous infections (see Kucers and Bennett, 1987). In fact, the number of reports demonstrating the medical usefulness of metronidazole numbers well into the thousands (McFadzean, 1986).

Because of their specific role in the development and production of Flagyl (metronidazole), May and Baker Ltd. was presented with the Queen's Award for Technological Achievement in 1982. It is virtually impossible to determine the world usage of metronidazole today, particularly in light of the expiration of most patents in 1974. However, one can form an idea by observing that between April 1984 and March 1985, a total of 8.6 tonnes of metronidazole preparations were used in the U.K. alone (McFadzean, 1986).

1.6.2 Spectrum of metronidazole activity

Metronidazole, 1-(β -hydroxyethyl)-2-methyl-5-nitroimidazole, is a nitroimidazole drug similar to tinidazole, nimorazole, ornidazole, carnidazole and secnidazole (Edwards, 1980; Kucers and Bennett, 1987). From the history and development of metronidazole, it is clear that this compound is highly toxic to organisms growing in anaerobic environments.

Metronidazole is active against some anaerobic protozoa. *T. vaginalis*, *E. histolytica*, *Giardia lamblia* and *Balantidium coli* all show some degree of susceptibility to metronidazole (Kucers and Bennett, 1987). Despite some initial reports that metronidazole could be used to treat leishmaniasis (Long, 1973; Jones, 1979) and trypanosomiasis (Davies, 1967), it appears that this compound has little or no effect on the responsible aetiological agents (Keithly and Langreth, 1983).

Investigations have clearly shown that metronidazole is active against most obligately anaerobic bacteria (Tally et al., 1981; Noble and Tally, 1984). Most

Bacteroides species, *Fusobacterium* species and *Clostridium* species are uniformly susceptible to metronidazole and other nitroimidazoles. Anaerobic organisms such as *Peptococcus*, *Peptostreptococcus* and *Eubacterium* species are generally susceptible to metronidazole, however occasional resistance is known (Tally et al., 1981; Dubreuil et al., 1984). The two medically important capnophilic organisms, *Campylobacter fetus* and *Haemophilus vaginalis* have also been shown to be susceptible to metronidazole (Chow et al., 1978; Pfeifer et al., 1978). Generally, most aerobic and microaerophilic bacteria show uniform resistance to metronidazole, however recent evidence suggests that certain facultative anaerobes are susceptible to metronidazole if grown under anaerobic conditions (see Chapter 4).

Resistance to metronidazole among susceptible species is rare (Müller, 1982), however resistant strains of *T. vaginalis* have been isolated from patients with refractory trichomoniasis (Thurner and Meingassner, 1978; Forsgren and Forssman, 1979; Heyworth et al., 1980; Lossick, 1981; Waitkins and Thomas, 1981; Kulda et al., 1982). Ingham et al. (1978) isolated a metronidazole resistant strain of *B. fragilis* from a patient who was on long term therapy with metronidazole for Chron's disease. No extrachromosomal DNA was found in this strain and there was no transfer of resistance determinants using filter mating techniques which have been successful for intraspecies transfer of clindamycin, erythromycin and tetracycline resistance in *B. fragilis* (Tally et al., 1981). Using radiolabelled metronidazole, it was shown that this resistant strain had a reduced ability to take up the drug (Tally et al., 1979). This was confirmed in a later study where metronidazole resistant *B. fragilis* strains were selected after mutagenic treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Britz and Wilkinson, 1979). Five metronidazole resistant mutants were isolated from different *B. fragilis* strains. In all cases, the mechanism of resistance was a reduced ability to take up the drug.

1.7 Mode of action of metronidazole

The mechanisms behind the mode of action of metronidazole have not been completely elucidated, however a good understanding is available. Müller (1983) described the essential action of metronidazole to occur in four steps: 1) entry of the drug into the cell, 2) reductive activation of the drug, 3) the toxic effects of the reduced product or products, and 4) release of the inactive end products.

1.7.1 Metronidazole entry

It is generally assumed that entry of metronidazole into the cell occurs via passive diffusion, however the supporting evidence is indirect and therefore active transport can not be totally ruled out (Ings et al., 1974). Using ^{14}C labelled metronidazole, Ings et al. (1974) showed that *T. vaginalis* cells rapidly accumulated the ^{14}C label. Non sensitive cells such as *Tetrahymena pyriformis* did not accumulate the ^{14}C label, although it was shown that metronidazole can diffuse into these cells. Various imidazoles and biologically active or inactive nitroimidazole derivatives in high concentrations do not inhibit this accumulation (Edwards, 1980; Müller, 1983). Because metronidazole undergoes metabolic modification after entry into sensitive cells, it has been suggested that this modification decreases the intracellular concentration of the unchanged drug and thus forms a transmembrane concentration gradient which promotes further drug uptake (Ings et al., 1974; Müller and Lindmark, 1976; Tally et al., 1978). Müller (1983) pointed out that because studies using ^{14}C labelled metronidazole showed that labelled products leave the cell, it is likely that the measured accumulation of the label in these studies underestimates the total drug entry.

1.7.2 Metronidazole activation

The fact that metronidazole is a prodrug is fortuitous, and was never envisioned when the drug was first synthesized and used (McFadzean, 1986). It has been suggested repeatedly that reduction of the nitro group of metronidazole is necessary for drug activation. The actual mechanisms of reduction of metronidazole have not been directly elucidated. Possible theories on how

metronidazole is reduced include direct electron transfer via reduced electron carriers, as well as active reduction via enzymatic routes. These possibilities are discussed in the next two sections.

Metronidazole reduction most likely occurs in single electron steps, assumed to lead firstly to the formation of a free radical anion, which can be reoxidized by oxygen to the original compound with the production of superoxide (Müller, 1983). Further reduction can yield a nitroso derivative, a nitroso free radical and a hydroxylamine derivative (Müller, 1983). These reduced intermediates are very short lived, and none of these compounds have been isolated. Using anoxic mammalian liver microsome preparations and electron paramagnetic resonance spectroscopy, Perez-Reyes et al. (1979) demonstrated the existence of the one electron free radical.

1.7.2.1 Role of electron carriers

O'Brien and Morris (1972) showed that when sub-lethal concentrations of metronidazole were added to exponentially growing *C. acetobutylicum* cultures, the drug rapidly disappeared from the culture medium. When metronidazole was added to crude extracts of *C. acetobutylicum*, hydrogen production was halted until no metronidazole remained in the assay flask. The production of CO₂ was not altered, indicating that the phosphoroclastic breakdown of pyruvate to acetyl-CoA continued. The effect of metronidazole on the uptake of hydrogen by the purified *C. pasteurianum* hydrogenase was also examined. It was shown that metronidazole caused no inhibition of the hydrogenase, and in fact extended the phase of rapid consumption of hydrogen. For each μmol of metronidazole supplied, approximately 3 μmol of additional hydrogen was utilized. These authors therefore concluded that, in *C. acetobutylicum*, metronidazole did not directly inhibit the hydrogen production, but inhibited the action of the hydrogenase by serving as an alternative and preferred acceptor of electrons from reduced Fd.

Non-enzymatic transfer of electrons to metronidazole was implied to occur in a hydrogenase linked assay system developed by Chen and Blanchard (1979). In

the reverse reaction, a purified hydrogenase consumes hydrogen gas forming reduced Fd (or flavodoxin), which in turn transfers electrons to metronidazole forming its reduced intermediate(s). Therefore the concentration of the electron carrier (Fd or flavodoxin) determines the amount of metronidazole that is reduced. After stopping the reaction, the amount of unreduced metronidazole was measured spectrophotometrically. Critical to the design and working of this assay are the facts that the reduction of metronidazole is irreversible and that once reduced, metronidazole loses its absorption peak at 320 nm.

Lockerby et al. (1984) clearly demonstrated the requirement of Fd for the activation of metronidazole. Cell free extracts of *C. pasteurianum* were capable of reducing metronidazole as measured by the uptake of hydrogen gas. The uptake of hydrogen gas only occurred in the presence of metronidazole. However, removal of Fd from the cell free extracts by DEAE cellulose treatment eliminated hydrogen uptake in the presence of metronidazole. These authors concluded that metronidazole preferentially scavenges electrons from reduced Fd, thereby depriving other Fd linked systems of essential reducing equivalents. The existence of a "metronidazole reductase" system that enzymatically transfers electrons from reduced Fd to metronidazole was suggested, however no evidence or speculation was provided concerning the components of this system.

1.7.2.2 Role of enzymes

The role of enzymes in the reduction of metronidazole has been postulated for a number years. Unfortunately, many reports simply cite the responsible enzyme or enzymes as being "nitroreductases" (Edwards et al., 1973; Rosenkranz and Speck, 1975; Wardman and Clarke, 1976; Tally et al., 1978; Chrystal et al., 1980; McLafferty et al., 1982). This term most likely arose from the fact that the nitro group of metronidazole needs to be reduced in order for activation, and may not refer to specific nitroreductase systems found in many microorganisms.

Since metronidazole inhibits anaerobic organisms and not aerobic organisms, it has been postulated that metronidazole interacts with enzymes present only in the biochemical pathways found of obligate anaerobes (Edwards et al., 1973; Ings

et al., 1974; Noble and Tally, 1984). Edwards and Mathison (1970) first noted that metronidazole inhibited hydrogen production in *T. vaginalis*, and similar results were found when metronidazole was added to cultures of *C. acetobutylicum* (O'Brien and Morris, 1972).

With particular reference to the clostridia, the production of hydrogen gas is the final step of a major pathway for the disposal of electrons. Electrons derived from the catabolism of pyruvate are transferred to oxidized Fd to form reduced Fd with the concomitant production of acetyl-CoA and CO₂ (see section 1.2.4). Electrons carried by reduced Fd are in turn transferred to protons to form hydrogen gas. Since the redox potential of metronidazole has been estimated to be very close to that of Fd (O'Brien and Morris, 1972), it can be postulated that enzymes capable of transferring electrons to Fd can also transfer electrons to metronidazole. In the clostridia the two principal enzymes that require Fd for complete functionality are the pyruvate-Fd-oxidoreductase and the hydrogenase.

The implication of pyruvate degrading enzymes in the activation of metronidazole was noted by Britz and Wilkinson (1979). Using chemical mutagenesis, these authors isolated metronidazole resistant mutants of *B. fragilis*. All of the metronidazole resistant mutants showed reduced levels of pyruvate dehydrogenase, which is an analogous enzyme to the pyruvate-Fd-oxidoreductase found in the clostridia (Narikawa and Nakamura, 1987).

Narikawa (1986) correlated pyruvate-Fd-oxidoreductase activity with metronidazole susceptibility. Metronidazole uptake, *p*-nitro benzoic acid reduction, pyruvate-Fd-oxidoreductase activity and metronidazole sensitivity, was determined for 41 different species covering 19 different genera. All of the obligate anaerobes tested and 4 of the facultative anaerobes were capable of reducing *p*-nitro benzoic acid. The ability to reduce *p*-nitro benzoic acid correlated directly with an ability to decrease the concentration of metronidazole in the culture medium. However, only the obligate anaerobes were identified as being susceptible to metronidazole, and this susceptibility correlated with pyruvate-Fd-oxidoreductase activity.

Lockerby et al. (1985) studied the effects of metronidazole reduction on the phosphoroclastic breakdown of pyruvate. It was shown that the addition of metronidazole to cell free extracts of *C. pasteurianum* caused an increase in acetyl phosphate production. It was also shown that Fd removal from the cell free extracts resulted in the complete loss of acetyl phosphate production. It was concluded that the role of the phosphoroclastic reaction with respect to metronidazole activation was to provide electrons through reduced Fd that would normally be evolved as hydrogen gas. Furthermore, stimulation of phosphoroclastic activity was presumed to be due to the fact that the reduction of metronidazole recycles Fd to its oxidized state more rapidly than the hydrogenase. This indicates that hydrogenase activity is the rate limiting step in the phosphoroclastic breakdown of pyruvate. Again, the presence of a "metronidazole reductase" system was assumed to carry out the transfer of electrons from reduced Fd to metronidazole, however no evidence was provided as to the components of this system.

In an elegant study Church et al. (1988) demonstrate that the bidirectional hydrogenase-1 of *C. pasteurianum* is in fact the "metronidazole reductase" that these authors (nee Lockerby) mentioned previously. Strong stoichiometric evidence was presented indicating that metronidazole replaces protons as the substrate for the hydrogenase enzyme. Together with reduced Fd, the hydrogenase transfers electrons to metronidazole yielding oxidized Fd and reduced metronidazole. In this case no hydrogen gas would be produced, which is in agreement with previous reports that showed hydrogen production was inhibited in the presence of metronidazole (Edwards and Mathison, 1970; O'Brien and Morris, 1972). A similar role for this hydrogenase enzyme has been shown in reducing 2-, 4-, and other 5-nitroimidazole drugs (Church et al., 1990).

1.7.3 Cytotoxicity

Because the biologically active reduction products of metronidazole have never been isolated, the actual mechanisms involved in the cytotoxic action of this drug remain largely unknown. Although the existence of multiple targets within

sensitive cells has been suggested (Buchner and Edwards, 1975; Carosi et al., 1977; Müller, 1983), an interaction with DNA is the most widely held explanation for metronidazole's cytotoxicity (Ings et al., 1974; Goldstein et al., 1977). Model experiments where metronidazole was reduced in the presence of various target macromolecules showed primary interaction with DNA (Edwards, 1977; LaRusso et al., 1977). Neither the unreduced drug nor the drug reduced prior to macromolecule addition reacted with DNA. Surprisingly, no interaction with RNA has been observed (Müller, 1983). Although there is some binding of the drug to nucleic acid (LaRusso et al., 1977), it is more important to note the occurrence of single strand and double strand breaks in the DNA and the release of nucleotide phosphates (Knight et al., 1978; Rowley et al., 1979, 1980; Edwards et al., 1980).

1.7.4 Inactive end products

The end products of reduction of metronidazole, such as acetamide (Koch et al., 1979) and 2-hydroxyethyl oxamic acid (Koch and Goldman, 1979), represent fragments of the parent molecule (Chrystal et al., 1980; Beaulieu et al., 1981; Müller, 1983). No cytotoxic activity has been demonstrated for any of these end products (Ings and Constable, 1975).

Chrystal et al. (1980) proposed that the amount of acetamide accumulated by the reduction of metronidazole is proportional to the amount of reduced toxic intermediate(s). This allowed for kinetic studies correlating the rate of metronidazole reduction by various bacteria with a decrease in the number of viable cells (Chrystal et al., 1980; McLafferty et al., 1982). The results suggested that the microbicidal activity of metronidazole depends not only on the capacity of the microorganism to produce the toxic intermediates, but also on the microorganism's specific susceptibility to these reduced toxic intermediates. In particular, the efficiency of a cell's DNA repair mechanisms partly determines its sensitivity towards metronidazole. Studies by Jackson et al. (1984) and Yeung et al. (1984), where the *in vitro* activity of metronidazole against DNA repair mutants of *E. coli* was measured, verified this hypothesis (see also Chapter 4).

1.8 Aims and overview of this thesis

C. acetobutylicum is an important industrial microorganism with tremendous potential for the fermentative production of solvents. Although this organism has been utilized for solvent production for a number of decades in this century, only recently have the mechanisms behind the regulation of these solvent producing pathways started to become clear.

The fundamental goals of this thesis are essentially divided into two parts. First, it was considered important to develop and utilize a suitable extraction and assay procedure for the measurement of nucleotide levels in *C. acetobutylicum* P262. As mentioned previously, fluctuations in nucleotide levels have been shown to be important in the regulation of other differentiating bacteria such as *B. subtilis*. Furthermore, ATP is the currency by which one measures energy in a cell and therefore following the concentration of this molecule through the course of a *C. acetobutylicum* fermentation was desired. While the content is far removed from the subject of microbiology, the second chapter describes the development of an analysis system for chromatographic data. The application of this system is demonstrated throughout the third chapter, where nucleotide profiles were obtained and analysed with an insight into how nucleotide levels affect or are affected by changes in the fermentation pattern of *C. acetobutylicum*.

The second part of this thesis shows a move from physiological studies into the realm of molecular genetic analysis of *C. acetobutylicum*. The biochemistry of the *C. acetobutylicum* fermentation clearly shows the importance of electron transport in determining the direction of carbon flow. Little is known about these electron transport genes, and the same is true for their regulation. The fourth chapter describes the development of a metronidazole based negative selection screening system for *C. acetobutylicum* electron transport genes in *E. coli*. The fifth chapter demonstrates the application of this system and the isolation of *E. coli* clones containing possible *C. acetobutylicum* electron transport genes which activate metronidazole. The sixth chapter describes the molecular genetic

analysis of *C. acetobutylicum* DNA from selected clones. Throughout this half of the study, efforts are made to relate the results to the activation of the medically important drug metronidazole.

Chapter 2

Development of a chromatographic analysis system with particular application for measuring nucleoside phosphate levels in *C. acetobutylicum* P262

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Chapter 2

Development of a chromatographic analysis system with particular application for measuring nucleoside phosphate levels in *C. acetobutylicum* P262

2.0 Summary

A chromatographic data acquisition and analysis system was developed for measuring the concentrations of ribonucleotides in bacterial cell extracts. The availability of multi-channel analog to digital converters provides a platform for application of this system to many types of gaussian analog output, which encompasses most analytical chromatographic techniques.

A manual integration system for the analysis of chromatographic data is described. The system features the ability to collect and store chromatographic data allowing for uninterrupted sequential runs to proceed. The data can be integrated many times using different calibration curves, permitting multiple analyses. The long term storage of data allows the user to assess changes in the system being employed, an important feature in chromatography systems where the columns and reagents are subject to constant change. The analog output produced by an absorbance monitor is passed to a non-inverting signal amplifier. This amplified signal is sent to an IBM PC where an analog to digital converter is used to digitize the data. A set of six computer programs which collect, store and analyze these data are discussed. This system was used to analyze the nucleotide content *C. acetobutylicum* P262 by strong anion-exchange HPLC.

2.1 Introduction

Since their introduction into universities and research establishments in the early 1960s, computers have been widely used by biologists (Ireland and Long, 1985). This has been facilitated by the use of analog to digital (A/D) converters, which take an analog input (a current or voltage) and convert it into a representative digital value. Because the analog signal is digitized into discrete values, the total number of values represents a subset of the total range of possible values generated by the analog source. The more bits available to digitize the analog signal, the greater the number of discrete values, and hence the greater the accuracy of the conversion. For example 8-bit resolution permits the decomposition of an analog signal into 256 (2^8) discrete units, whereas 12 bit resolution permits the same analog range to be divided into 4096 (2^{12}) discrete units. This translates into a resolution of 0.39% for an 8-bit A/D converter and 0.02% for a 12-bit A/D converter. In comparison, a good quality chart recorder has a mechanical resolution around 0.1 to 0.2% (Beynon, 1985).

High performance liquid chromatography (HPLC) is an important technique in the biological sciences. Most HPLC applications produce output via a detector that generate an analog signal. This analog signal is typically directed to a chart recorder which may be associated with an integrator. A number of automated microprocessor-controlled integration systems are commercially available for HPLC applications. In general, these automated analysis systems rely on the presence of a flat and stable baseline and peaks that are sharp and gaussian. When relying on quantitation by peak height, adherence to these parameters is of utmost importance. Quantitation by peak area is more flexible, but is still sensitive to sloping baselines and trailing peaks, which are common when a gradient mobile phase is used.

Many methods for separating and quantitating nucleoside phosphates by anion exchange HPLC have been reported (Hartwick and Brown, 1975; Khym, 1975; Payne and Ames, 1982; Freese et al., 1984a). Bacterial cell extracts contain many UV absorbing compounds (Khym et al., 1977; Khym et al., 1978). Separation of

nucleoside phosphates in these complex bacterial extracts by anion exchange HPLC generally involves the use of a gradient buffer system, which inherently results in a chromatogram with a sloping baseline. Nucleotide triphosphates elute on or just prior to the most sloped region of the baseline. Measurement of nucleotide phosphate levels in cell extracts of *C. acetobutylicum* was a goal in this study. Therefore it was necessary to develop a reliable chromatographic analysis package for separation and quantitation of nucleotide triphosphates in these cell extracts

This chapter presents a system for interfacing a HPLC detector to an IBM PC or compatible microcomputer via an A/D converter, and a set of six computer programs which collect, store and manipulate the HPLC data.

These programs can be conveniently utilized to manually integrate complex chromatograms. The programs are written to be highly flexible and can be applied to many different types of chromatographic data (including other types of flow cell monitoring chromatography as well as densitometry). Raw data is collected, stored on disk and can be immediately integrated or integrated at a later stage, allowing for sequential runs to proceed without data analysis. Once integrated, the quantitation data is stored and the chromatogram can be re-integrated at any time.

2.2 Materials and Methods

2.2.1 Instrumentation

A diagram of the chromatographic and computer hardware systems is shown in Fig. 2.1. The HPLC system (Beckman Instruments Inc., Berkely, CA, USA) consisted of two solvent delivery modules (model 114M), a flow rate and gradient controller (model 421), a mixer-organizer (model 340), a constant volume injector (model 210A) and a fixed wavelength absorbance detector (model 160). The analytical column (25 mm long X 4.6 mm i.d.) used on this HPLC system contained the strong anion-exchanger Partisil 10-SAX (Whatman Inc., Clifton, NJ, USA). The analytical column was protected by a guard column

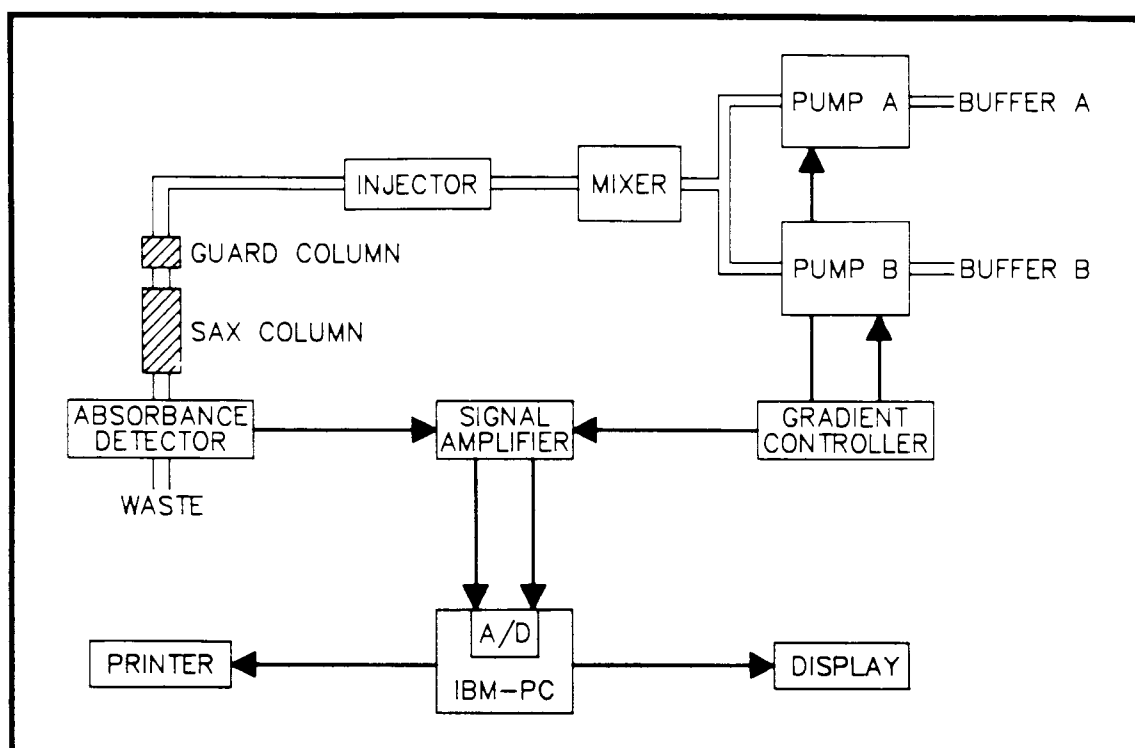


Figure 2.1 A diagrammatic representation of the computer and HPLC hardware systems.

containing Pellionex SAX media. The details of the chromatographic conditions are described in section 3.2.5.

2.2.2 Application

The HPLC-computer integration system was designed for evaluating the ribonucleotide composition of cell extracts of *C. acetobutylicum* P262. The nucleotides of interest were ATP, CTP, GTP and UTP, as well as the 5'-di and 5'-monophosphates of these purine and pyrimidine bases. Nucleotide extracts of *C. acetobutylicum* cells were prepared as described in section 3.2.4.

2.2.3 Signal amplifier

The low output signal (0-20 mV) from the fixed wavelength detector necessitated the use of a signal amplifier. The circuit shown in Fig. 2.2 was used to amplify

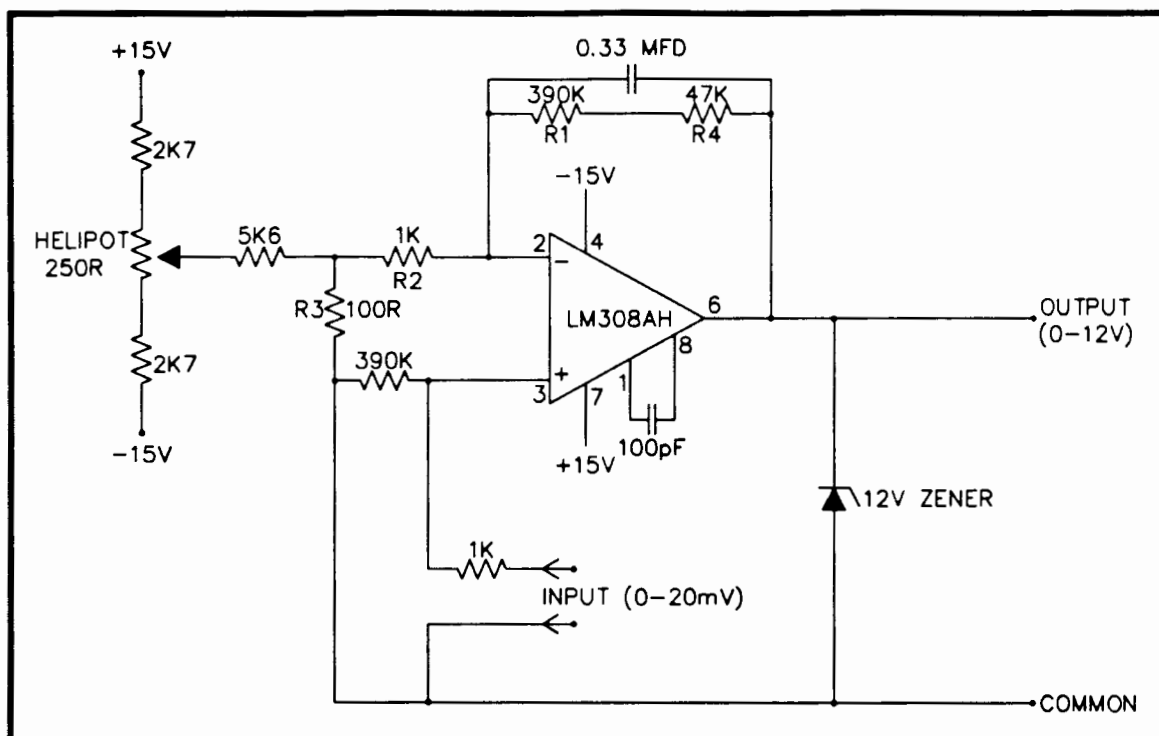


Figure 2.2 A diagram of the circuit used to amplify the 0-20 mV analog signal produced by the HPLC absorbance detector. The LM308AH operational amplifier was manufactured by National Semiconductor.

the output signal produced by the model 160 detector. The circuit is a non-inverting amplifier and its gain was determined by the ratio:

$$\frac{R1 + R4}{R2 + R3} + 1$$

By inserting the shown resistor values, the gain of the amplifier was calculated to be approximately 400. The 250 Ω HELIPOT and associated components were used to provide an offset facility and determine baseline levels. The 12 V zener diode connected to the output of the amplifier ensured that the signal to the computer did not go below -0.6 V or above 11 V.

2.2.4 Computer hardware

The computer system consisted of an IBM PC with 640 kbyte RAM, a green monochrome screen, a Data Products 8010 dot matrix printer, a Hercules compatible monochrome graphics/printer adapter and an Eagle Electronics (PC-26) 16 channel 12-bit analog to digital converter card.

2.2.5 Interfacing

The amplified analog signal from the HPLC was connected to channel one of the A/D card in the IBM PC. The HPLC controller has four internal relays which can be programmed to be open or closed during the chromatographic run. One of these relays was used to send a 9 V signal (external trigger) to channel zero of the A/D card which initiated the computer to sample the incoming data on channel one.

2.2.6 Language, files and compilation

Two versions of these programs were written. The first version was written for the Hercules HBASIC interpreter. This allowed for normal IBM BASIC graphics commands to be implemented on the monochrome monitor; however this version was found to be too slow for routine use. Although it has not been tested, this version could be compiled and run on an IBM PC with a color graphics adapter and monitor, with little or no modification.

The second version of these programs was written in IBM BASIC using edition 1.1 of the Hercules GRAPH-X on screen utilities package (Hercules Computer Technology Inc., Berkeley, CA, USA), and was compiled using version 1.0 of the Microsoft QuickBASIC compiler (Microsoft corporation, Redman, WA, USA). The Hercules GRAPH-X subroutines were CALLED from within the main program and the GRAPHIX library was linked to the compiled object modules using the Microsoft Link program provided with the QuickBASIC compiler. The complete source code for this second version of these is located in appendix F.

2.3 Algorithm

Six computer programs were written to handle the interfacing and data collection from the HPLC: STARTUP, HPLC, MANINT, MANINTST, DISPLAY and CALIBRAT.

The STARTUP program was included to load the compiler runtime module and the GRAPHIX runtime module. This program puts a logo on the screen and CHAINED the HPLC program.

The HPLC program was the menu generating program that provided the main entry and exit mechanism for the software system. Menu choices were (i) data collection and manual integration, (ii) manual integration of a stored chromatogram, (iii) display a stored chromatogram, (iv) record calibration chromatograms and (v) exit to DOS.

MANINT (data collection and manual integration) and MANINTST (manual integration of a stored chromatogram) were the two quantitation programs in the series. MANINT was the data collection - integration program and the user could select if the raw data was to be immediately integrated, or integrated at a later time. The MANINTST program was used for integrating these stored chromatograms, as well as re-integrating stored chromatograms.

Selecting the MANINT program from the HPLC menu initialized the 8255 programmable peripheral interface on the PC-26 A/D card. It prompted the user for the name of a file for data storage and asked if an external trigger was being used to initiate sampling. The time versus absorbance axes were drawn on the display and sampling of the A/D card began. If an external trigger was being used to initiate data input, channel zero of the A/D card was sampled until a value greater than 2 500 (approximately 7 V) was read. Once channel zero was high (> 7 V) sampling of the incoming data on channel one was initiated. The digitized data was stored in an integer array and the corresponding sample time was stored in a double precision array. The display was set to mimic a chart recorder and the incoming data was actively plotted on the screen during the course of the chromatographic run. To encompass the part of the chromatogram of interest for this application, the total sampling time was set to 30 min. The maximum sampling rate of the PC-26 card was 2 500 Hz and this sampling rate would generate an impractical 4.5 million samples. Therefore the program was designed to sample channel one every 0.36 sec., which yielded approximately 5 000 samples per chromatogram. For nucleotide analysis, the average peak width at the baseline was approximately 2 min. Therefore, each peak was characterized by approximately 300 samples which was more than adequate for integration. During a run, the date, time of day, runtime and number of samples

taken were displayed in a menu area at the bottom of the display. After the 30 min sampling period the raw data was stored on disk along with the date, injection time (real time) and the total number of samples taken. The user could then continue with manual integration, perform another chromatographic run or exit to the HPLC program. If the option to quantitate the data was chosen, the chromatogram remained displayed and manual integration continued as described below.

MANINTST prompted the user for the file name containing the raw data. The data was read from disk, the absorbance versus time axes were drawn and the data was plotted. From this point on, the MANINT and MANINTST programs were identical.

An integration command menu describing the control keys appeared below the displayed chromatogram. A vertical marker line could be moved left or right using the cursor keys on the numeric keypad. This vertical marker was used to locate the peak starts and peak ends. When this was positioned at a peak start and the 'S' key was depressed, a mark was made on the chromatogram indicating a selected peak start. Similarly, if the 'E' key was depressed a mark was made on the chromatogram indicating a selected peak end. Other options included CLEAR entry which removed an incorrectly selected peak start or peak end, REDO from start which removed all selected peak starts and peak ends, QUIT integration which abandoned the integration procedure and returned control to the HPLC program and finally PROCESS data which integrated the selected peaks.

When the PROCESS data option was chosen, selected peak starts and peak ends were sorted with respect to time. The starts and ends were checked for pairs and checked that they were in the correct order. If no errors were found, the area under each peak as well as the peak retention time were calculated. The peak retention time was determined using the Savitzky and Golay (1964) method of calculating a smoothed first derivative. The baseline was calculated using the 'rubber band' technique of Caesar and Klier (1974). The peak area was

determined as the sum of trapezoids from data point to data point between the selected peak start and peak end.

Peak start and peak end marks were removed from the display and the retention times were written above each selected peak as they were calculated. Upon completion of these calculations, a hardcopy of the chromatogram was obtained using the graphics dump facility provided with the Hercules GRAPH-X package. A short assembly language subroutine, PRTSCH, was written to invoke this graphics dump from within the program.

The calculated retention times were then compared to the retention times of known compounds (standards) which had been stored on disk using the CALIBRAT calibration program. If the calculated retention time fell within a pre-set threshold level surrounding the mean retention time for the standard, the peak was presumed to be the same as the standard. Using the stored standard curve, the calculated peak area was extrapolated to the same units of measurement used when creating the standard curve. These data were printed in tabular form on the dot matrix printer and all quantitated data were stored on disk. The user then had the option to repeat the program or return to the HPLC program.

The DISPLAY program read the raw data from disk and drew the chromatogram on the screen. If the chromatogram had been integrated, this data was also read from disk and was presented in tabular form on the second graphics page of the Hercules card. By toggling between graphics pages, both the chromatogram and the quantitated data could be viewed on the display. A hardcopy of the chromatogram and the quantitated data could also be obtained if desired. The user then had the option to view another chromatogram or return to the HPLC program.

For the entire package to work a calibration file must be created using the CALIBRAT program. This file, contained the names and retention times of known compounds, and the standard curves (least square regression lines) of the

quantity of material versus the peak area for each known compound. A mixture of known compounds was injected into the HPLC and recorded by the CALIBRAT program. At the end of the chromatographic run, the user selected peak starts and peak ends from which the retention times and peak areas were calculated. In this respect the CALIBRAT program was similar to the MANINT and MANINTST programs. The user was prompted for the peak (compound) name and the quantity injected. The quantity injected could be expressed in any units the user desired. A hardcopy of the chromatogram as well as the entered data could be obtained at this point. This process was repeated and all the information entered was stored in a temporary file. When desired, this data could be processed and mean retention times and least square regression lines calculated. The standards file used by the MANINT and MANINTST programs was updated with these new values. This was a very powerful feature of this system, since it allowed the user to re-integrate chromatograms using different calibration files. If desired, the user had the option to halt the calibration procedure and save the temporary file, thus freeing the computer for other uses. Upon starting the CALIBRAT program, the presence of a saved temporary file was searched for. The user also had the option to abandon the calibration procedure in which case the temporary file was destroyed. The date of calibration was stored in the standards file and was noted on chromatograms integrated with the MANINT and MANINTST programs.

2.4 Implementation

The programs and system described above were used to analyze the ribonucleotide composition of cell extracts of *C. acetobutylicum* P262. The chromatographic run time was approximately 45 min, with the nucleotide di- and tri-phosphates eluting from 20 min onwards. Since the beginning of the chromatogram was not of interest, sampling of absorbance data was initiated from the model 421 controller (external trigger) 15 min after injection of the sample. Absorbance was measured at a wavelength of 254 nm, which is near optimal for most nucleotide phosphates (Freese et al., 1984a). The absorbance

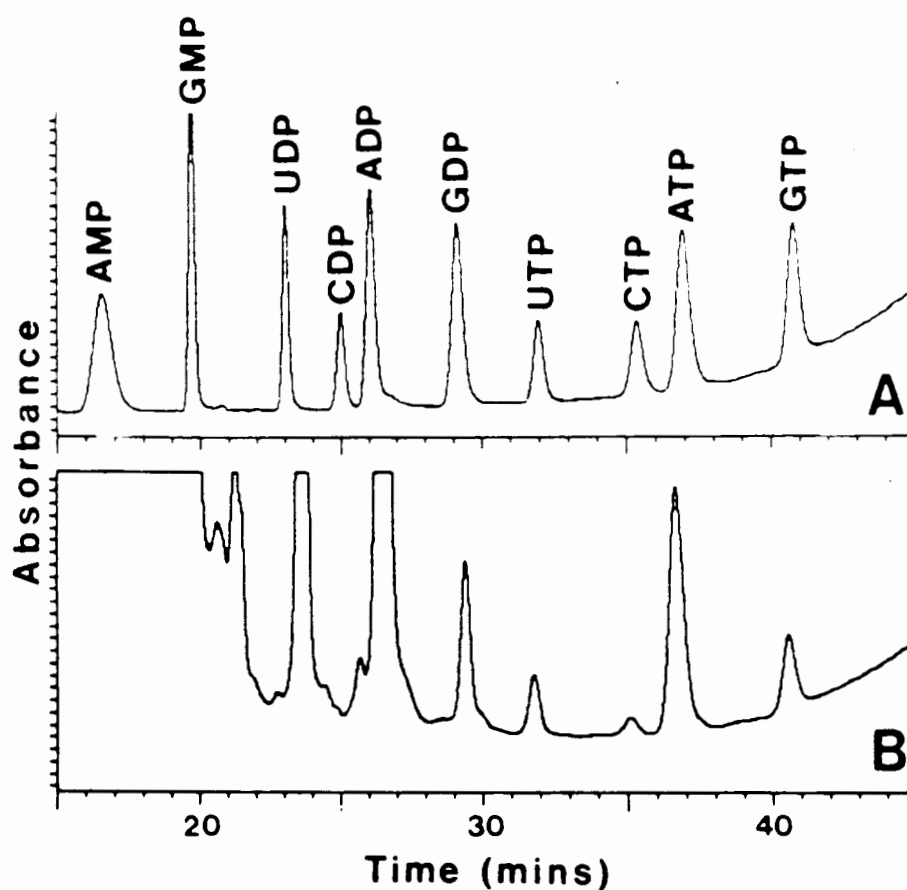


Figure 2.3 Chromatograms of (A) a standard mixture of nucleotides and (B) an extract of *C. acetobutylicum*. The absorbance (0.04 OD units = full scale) was measured at 254 nm.

range of the model 160 detector was set so that 0.04 OD units equaled the full scale value on the analog output (20 mV).

The computer section was initiated from DOS through the STARTUP program which gives control to the menu generating HPLC program. Data collection was carried out with the MANINT program, unless the system was being calibrated, in which case the CALIBRAT program was utilized. Data collection started at 15 min after injection when the external trigger from the 421 controller went high (approximately 9 V). At 45 min, data collection was stopped and the raw data stored on disk. This was followed by manual integration which generated the quantitated data which was also stored on disk. Fig. 2.3 shows a typical hardcopy output of a chromatogram obtained on the dot matrix printer. All data

stored on disk could be accessed for re-integration by using the MANINTST program or could be viewed with the DISPLAY program. If the calibration program, CALIBRAT, had been run, the standards file was updated and control was returned to the HPLC program. Access to DOS was obtained through the HPLC program, thereby assuring restoration of the graphics card and the operating system to their default values.

2.5 Discussion

A program for analyzing chromatograms has been written for use on a mainframe computer (Caesar and Klier, 1974). Although this program was originally developed for use with gas chromatography, the algorithm is applicable to liquid chromatography. Furthermore, Freese et al. (1984a) reported a full spectrum analysis package for analyzing liquid chromatograms on a DEC PDP 11/10 microcomputer (Digital Equipment Corp., Maynard, MA, USA) which allows for the manual integration of data. These two algorithms were used to form the basis of a general purpose data collection and manual integration package for use with analog output chromatography systems.

In designing this system, it was important to maintain accuracy while being frugal. The use of a 12-bit A/D converter divides the analog signal produced by the fixed wavelength detector into 4096 (2^{12}) discrete units. This yields a resolution of approximately 0.02% (Beynon, 1985), which was more than adequate for this application. Since microcomputers have become standard items in the scientific laboratory, this system did not require a large capital expenditure. The equipment required to assemble this system cost far less than the purchase price of an automatic integrator.

In any chromatography system, peak identification is of utmost importance. Peaks are often identified by comparing their retention times with retention times of known compounds. In order to identify compounds with some degree of certainty, more than one identification method should be employed. In

biological extracts where there are numerous compounds present, retention time comparison alone is not an adequate means of identification (Brown et al., 1980).

The system described here does not provide a foolproof computer analysis for the identification of unknown compounds. It is assumed that the user fully understands the analytical methods being employed. Although these programs used retention time to identify peaks, this identification was only tentative. Fractions were routinely collected from the absorbance detector and a full spectrum scan was performed for comparison to scans of known compounds. Not only did this provide a method of peak identification, it also allowed for the determination of peak purity. If the scan demonstrated the presence of a co-eluting compound that absorbed strongly at or near 254 nm, the quantitation data for that peak was assumed invalid. In this system, the user can choose which peaks are to be integrated by selecting the appropriate peak starts and peak ends. Therefore, preliminary identification is carried out by the user prior to any retention time comparison by the computer. This is a major advantage of this system in contrast to automatic integrators, since it requires the user to make the initial decision as to peak identification.

Once a peak has been identified, it is normally required to quantitate the amount of material represented by that peak. The quantity of the eluted compound is generally expressed in terms of some property of the curves produced by the detector. Peak area was chosen to measure the quantity of the substance represented by a peak since it was found to be more accurate than peak height. Peak area is a widely used method for quantitation of chromatographic data and many techniques are available for estimating this value. Peak area was estimated geometrically by summation of the trapezoids formed from data point to data point produced by digitizing the amplified analog signal of the absorbance detector.

The system was routinely calibrated by injection of at least seven different concentrations of a standard nucleotide mixture containing the 5'-di and 5'-triphosphates of adenosine, cytosine, uridine and thymidine. In an experiment

where the standard curves calculated by the CALIBRAT program were challenged with 40 different known quantities of nucleotides, the mean deviation of estimated concentration versus known concentration was 3.68% of the known concentration. Furthermore, the largest deviation of an estimated concentration from the known concentration in an individual case was 6.51% of the known concentration.

Least squares regression line calculations (Snedecor and Cochran, 1980) were used in the CALIBRAT program to derive the standard curves. The correlation coefficients for peak area versus amount were found to be 0.94, 0.93, 0.96 and 0.91 for ATP, GTP, CTP and UTP, respectively. Similarly high (>0.9) correlation coefficients were found for the nucleotide di-phosphates. It was found that all calibration curves were linear in the absorbance range (0.04 OD units full scale) used on the model 160 detector. Since access to an automatic integration system was not available, an appropriate experimental comparison could not be performed. It is believed that the advantages of this manual analysis system far outweigh the conveniences of automated integration for analyzing complex biological materials. It has been reported that manual integration is more reliable when compared to automatic integration (Freese et al., 1984a) and this conclusion is supported by these results.

Chapter 3

Nucleoside triphosphate levels in *C. acetobutylicum* P262 wild type, sporulation deficient and solvent deficient strains

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Chapter 3

Nucleoside triphosphate levels in *C. acetobutylicum* P262 wild type, sporulation deficient and solvent deficient strains

3.0 Summary

The role of GTP and other ribonucleoside phosphates in the differentiation of *C. acetobutylicum* and two sporulation deficient mutants was investigated. The clostridial stage (*cls-1*) mutant was unable to produce the clostridial stage, solvents, granulose, capsules or endospores, whereas the sporulation deficient (*spo-1*) mutant produced the clostridial stage, solvents, granulose and capsules, but did not form a forespore septum. The nucleoside triphosphate profiles of the wild type and *spo-1* mutant were similar and were characterized by a trough in nucleotide levels which occurred just prior to the pH break point, the onset of the stationary growth phase, clostridial stage formation and the transition from the acidogenic to the solventogenic phase. The nucleoside triphosphate concentrations during the exponential growth phase were much lower than those found during the stationary phase. The nucleotide levels in the *cls-1* mutant during the exponential phase were comparable to those observed in the wild type and *spo-1* mutant. However, the nucleotide levels in the *cls-1* mutant did not increase during the stationary growth phase. The involvement of nucleotide levels, particularly that of GTP, in the differentiation of *C. acetobutylicum* was indicated by the effect of inhibitors, which have been shown to decrease ribonucleotide levels in other organisms and cause an increase in sporulation.

3.1 Introduction

In *B. subtilis* sporulation occurs when the cells encounter certain adverse nutritional conditions which no longer allow rapid growth. Nutrient deprivation causes the cells to divide asymmetrically, and the two membranes separating the smaller cell compartment from the larger mother cell, envelope the smaller compartment thus producing a forespore. Synthesis of spore specific proteins is derepressed which may be a result of an altered metabolic environment within the forespore caused by a membrane that does not allow for normal active transport (Freese, 1981). Freese and co-workers developed the theory that there is ultimately one compound of low molecular weight which in combination with a protein, suppresses sporulation. The theory being that when the concentration of this compound reaches critical levels, sporulation is induced.

Early studies analyzed the initiation of sporulation by using sporulation minus mutants and chemical inhibitors. Mutants that were auxotrophic for certain amino acids led to the conclusion that amino acid limitation, which initiated sporulation, also evoked the stringent response (Ochi et al., 1981). This response included the synthesis of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), which resulted in a decrease of GTP and GDP (Lopez et al., 1981). Ochi et al. (1981) showed that relaxed mutants (*relA*) which lacked the stringent response did not demonstrate a sufficient decrease in the level of GTP to initiate sporulation. This evidence implicated GTP as the low molecular weight compound that suppresses sporulation in *B. subtilis*.

GTP was further implicated as the trigger for sporulation by the observation that sporulation could be induced in a nutrient rich environment by the partial reduction in synthesis of purine nucleotides (Freese et al., 1978). This condition of sporulation was achieved by purine removal in leaky purine requiring mutants or by the addition of inhibitors of the purine biosynthetic pathway. A preferential decrease of GTP, but not ATP, was observed in the sporulating cultures. It has been proposed that the partial starvation for purine creates a new

biosynthetic balance which is conducive to the positioning of a septum in a polar rather than a central position within the cell (Mitani et al., 1977).

A similar decrease in GTP or S-adenosylmethionine content was shown to be associated with differentiation (meiosis and sporulation) in *Saccharomyces cerevisiae* (Freese et al., 1984b). Aerial mycelium formation in *Streptomyces* MA406-A-1 has also been linked with a decrease in GTP content (Ochi, 1986).

In some strains of *C. acetobutylicum*, such as the industrial strain P262, the onset of solvent production and sporulation is characterized by a change in morphology from a highly motile, acid producing, vegetative rod to a swollen, granulose containing, phase-bright, cigar shaped, solvent producing, 'clostridial' cell (Long et al., 1983; Jones and Woods, 1986a). An understanding of the triggers which lead to the onset of solvent production and differentiation is important for developments aimed at improving the acetone butanol fermentation.

Since the role of GTP and other ribonucleoside phosphates in the differentiation of *C. acetobutylicum* has not been reported prior to this investigation (Santangelo et al., 1989) a study into the changes in ribonucleoside phosphate pools of the wild type strain P262 and two sporulation deficient mutants was undertaken. The clostridial stage (*cls-1*) mutant is unable to form the clostridial stage and can not produce solvents, capsule, granulose or endospores (Jones et al., 1982). The second mutant (*spo-1*) forms the clostridial stage and produces solvents, capsule and granulose but does not form a forespore septum (Jones et al., 1982).

3.2 Materials and Methods

Commonly used materials and techniques are presented in the Appendices. The details provided below are included for completeness.

3.2.1 Bacterial strains

The wild-type *C. acetobutylicum* strain P262 was obtained from National Chemical Products (NCP), Germiston, South Africa, and has been described

previously (Jones et al., 1982). The *cls-1* and *spo-1* mutants were isolated from this strain by mutagenesis with ethyl methanesulphonate as described by Jones et al. (1982).

3.2.2 Media and culture conditions

The *C. acetobutylicum* strains were grown in the *C. acetobutylicum* minimal medium (CAMM) of Long et al. (1983), with the glucose concentration reduced from 60 g l⁻¹ to 50 g l⁻¹ and the nitrogen source changed from ammonium phosphate to ammonium nitrate (7.2 g l⁻¹). The clostridial basal medium (CBM) of O'Brien and Morris (1971) was inoculated with spores activated by heat shock at 75°C for 2 min and then cooled on ice for 45 sec. A 5 µl sample of the heat shocked spore suspension was used to inoculate 10 ml of medium. Cultures were incubated at 34°C until the cells reached mid exponential phase (OD₆₀₀ 0.45 to 0.50). CAMM (400 ml) was inoculated with 40 ml exponential phase cells harvested from CBM and washed once with distilled water. Cultures were incubated at 34°C and shaken gently every 6 h. All manipulations and incubations were carried out under stringent anaerobic conditions in an anaerobic glove box containing an atmosphere of oxygen-free N₂, CO₂ and H₂ (85:10:5 by volume).

3.2.3 Growth, morphology and end product determination

Total cell counts, clostridial stage counts, forespore counts and spore counts were determined with a Thoma counting chamber (Weber Scientific International, UK) and a Zeiss photomicroscope fitted with phase- and interference-contrast optics. Growth was measured turbidometrically at 600 nm with a Corning colorimeter (model 252) using Hungate tubes. Acetate, butyrate, acetone, butanol and ethanol were determined by gas chromatography on a Hewlett Packard 5880A gas chromatograph fitted with a flame ionization detector, using 2-propanol as an internal standard. The column and running conditions have been described previously (Long et al., 1984a).

3.2.4 Preparation of nucleotide extracts

Nucleotide extracts were prepared from *C. acetobutylicum* cells grown in CAMM. The extraction methods of Olempska-Beer and Freese (1984) and Payne and Ames (1982) were combined and modified for use with *C. acetobutylicum*. Cells (100 ml) were rapidly collected on a 100 mm diameter nitrocellulose filter (Schleicher and Schüll BA 84 or AE 95) by vacuum filtration. The filter was placed cell side down in a glass Petri dish containing 5 ml of ice-cold 3 M formic acid saturated with 1-butanol. After a 30 min extraction period, the liquid was removed and the filter and Petri dish were washed twice with ice-cold sterile water. The above manipulations were carried out in an anaerobic glove box, and all solutions were pre-reduced for at least 24 h. The three liquid fractions were combined, removed from the anaerobic chamber and adjusted to pH 8.8 with 25% (w/v) ammonia solution. The salt concentration of the extract was increased to 1 M by the addition of 3 M ammonium acetate, pH 8.8. Debris was removed by centrifugation at 40 000 X g for 20 min. The supernatant was applied to a column (1.5 cm long X 1 cm i.d.) packed with Affi-Gel 601 (Bio-Rad Laboratories, Richmond, CA, USA) which had been equilibrated with 1 M ammonium acetate, pH 8.8. The absorbance of the effluent was monitored at 254 nm using an Isco flow cell monitor (model UA5 fitted with a type 6 optical unit) and unwanted and interfering components were washed through the column with a mobile phase of 1 M ammonium acetate, pH 8.8. When the A_{254} of the effluent reached baseline, the mobile phase was changed to 100 mM acetic acid to initiate elution of the nucleotides. The desired sample which contained the nucleotides (about 10 ml) was collected and freeze-dried. The freeze-dried sample was resuspended in 100 μ l of water, centrifuged and assayed by HPLC. The reliability of this extraction method was routinely tested by performing duplicate extractions where a known concentration of a standard nucleotide mixture was added to one sample just prior to the formic acid treatment (Freese et al., 1984a).

3.2.5 Chromatographic conditions

The Partisil-10 SAX column described in section 2.2.1 was operated at room temperature at a flow rate of 1 ml min⁻¹. A 50 µl sample volume was injected and the nucleotides were separated using a multi-step gradient profile. The initial buffer (A) contained 7 mM potassium dihydrogen phosphate which was adjusted to pH 4 with HCl. The high ionic strength buffer (B) used to elute the nucleotides contained 0.5 M potassium dihydrogen phosphate and 0.5 M sodium sulfate, and was adjusted to pH 5 with potassium hydroxide. After injection, the mobile phase contained 100% buffer A for 12 min. A linear gradient was employed such that the mobile phase contained 50% buffer B after another 21 min (33 min run time). The slope of the gradient was then increased such that the mobile phase contained 100% buffer B after an additional 10 min (43 min run time). The mobile phase was maintained at 100% B for another 7 min (50 min run time), after which the mobile phase was changed to 100% buffer A in 5 min. The column was equilibrated at 100% A for 20 min prior to injection of the next sample. After 5 consecutive runs, the column was regenerated by the passage of 150 ml of 0.5 M sodium sulfate, pH 2 with sulfuric acid, followed by 50 ml of buffer B. This removed highly ionized, strongly bound impurities. Once a week the column was washed with 100% HPLC grade methanol to remove adsorbed organic impurities. The salts used to prepare the above buffers were all Aristar grade (BDH chemical) and were dissolved in 18 MΩ water (Milli-Q).

3.2.6 Analysis of nucleotide extracts

The computer-HPLC data collection and analysis system used to analyze the nucleotide composition of the extracts has been reported previously (Santangelo, 1988) and is described in Chapter 2. All experiments were performed at least three times, with similar results; representative data are presented.

3.2.7 Nucleotide synthesis inhibitors

Decoyinine and psicofuranine were generously donated by Dr. R.L. Keen, Upjohn Company, Kalamazoo, MI, USA. Caffeine and 3-methylxanthine were obtained from the Fluka Chemical Company. Theobromine, theophylline,

6-mercaptopurine, 2-amino-6-mercaptopurine and 6-azathymine were purchased from the Sigma Chemical Company. Since CAMM supports a near maximum spore titre for *C. acetobutylicum* P262, it was an unsuitable medium for inhibitor studies where an increase in sporulation was to be determined. The tryptone, yeast extract, glucose medium (TYG) of Hongo et al. (1968), which resulted in a lower sporulation level, was therefore used for these studies. The inhibitors were added during the mid exponential growth phase, when the OD₆₀₀ of the culture was between 0.40 to 0.45. Percentage sporulation was determined after 48 h.

3.3 Results

3.3.1 Growth, physiology and morphology

The ABE fermentation profile typical of *C. acetobutylicum* P262 in CAMM is shown in Fig. 3.1(a,b). Cells transferred to this medium show a characteristic 15 h lag phase after which cell numbers begin to increase (Long et al., 1983). The exponential growth phase, from 15 to 35 h, was characterized by highly motile, rod-shaped cells. During this stage, glucose consumption resulted in acid production. The pH break point occurred when the acid concentration reached a maximum of approximately 4 g l⁻¹ (between 30 and 35 h). The break point was associated with the onset of the stationary growth phase and clostridial stage formation. Just prior to the pH break point, the cell morphology began to change from motile rods to phase-bright, granulose filled clostridial cells, and by 40 h 90 to 100% of the cells had converted to the clostridial stage. The physiological changes associated with the pH break point included a decrease in acid concentration, initiation of solvent production, and an increase in the rate of glucose consumption. After 45 h, the acid concentration stabilized at approximately 2.7 g l⁻¹. Glucose exhaustion occurred at approximately 50 h and shortly thereafter solvent levels reached a peak of approximately 10 g l⁻¹. At this time a maximum number of cells containing forespore septa (60%) was observed, as well as a small percentage of mature spores (15%). The highest proportion of

phase-bright spores (approximately 50%) was observed at the end of the fermentation (67 h).

A similar fermentation profile for the *spo-1* mutant is shown in Fig. 3.1(c,d). The exponential phase was characterized by motile rods, glucose consumption and acid production. The pH break point (pH 5.3) was reached between 35 and 40 h and was associated with a peak in acid levels. Stationary phase occurred after approximately 40 h and by 55 h 90 to 100% of the cells were phase-bright clostridial forms. Solvents were first detected immediately after the pH break point and reached a maximum of 9.6 g l⁻¹ at the end of the fermentation (67 h). The major difference between this and the wild type fermentation was that no forespore septa or mature spores were observed, indicating that the *spo-1* mutant was an early sporulation mutant (*spo0*). Minor differences involving for example maximum cell number, time of peak in motility, and residual glucose levels were observed between the fermentation profiles of the P262 wild type and the *spo-1* strains.

The *cls-1* mutant showed a normal motile, acid producing, glucose consuming exponential phase, but the switch to solvent production did not occur and no clostridial stage cells were observed (Fig 3.1e,f). After approximately 35 h the rate of glucose consumption slowed and acid levels continued to increase. By 55 h the fermentation ceased and virtually no solvents were produced (<0.25 g l⁻¹). Only 30% of the available glucose was utilized and approximately 7 g acids l⁻¹ were produced.

3.3.2 Nucleotide profiles

The nucleoside triphosphate profile for the wild type *C. acetobutylicum* P262 strain showed that throughout the fermentation ATP was the most abundant nucleoside triphosphate followed by UTP, GTP and CTP (Fig 3.2a). The nucleoside triphosphate concentrations during the exponential growth phase (15 to 35 h) were much lower (by as much as 40-fold in the case of ATP) than the values obtained during most of the stationary growth phase (40 to 50 h). From

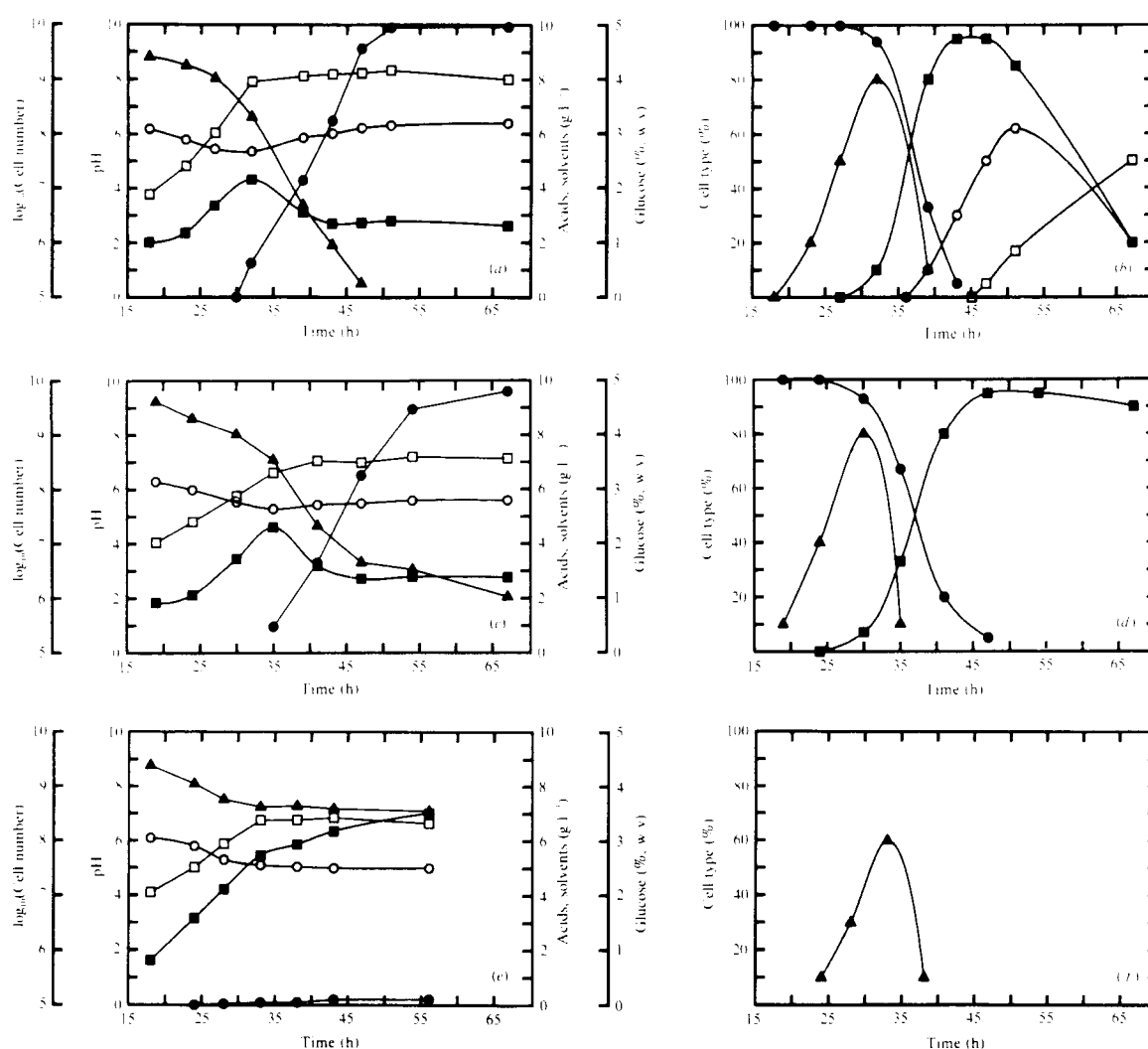


Figure 3.1 Morphological and physiological profiles for *C. acetobutylicum*. (a,b) Profiles for the wild-type strain P262; (c,d) profiles for the *spo-1* mutant; (e,f) profiles for the *cls-1* mutant. Symbols for graphs (a), (c) and (e) correspond to the following: \blacktriangle , glucose concentration (% w/v); \blacksquare , total acids (g l^{-1}); \bullet , total solvents (g l^{-1}); \square , \log_{10} cell number; \circ , pH. The plots in (b), (d) and (f) are expressed as percentages of the total cell count, and the symbols correspond to the following: \blacktriangle , motility; \blacksquare , phase bright clostridial cells; \bullet , phase dark rods; \circ , forespore containing cells; \square , mature spores. In (f), only motility is shown as the *cls-1* mutant remained as 100% phase dark rods throughout the fermentation.

17 to 23 h there was a gradual decrease in the concentration of all the nucleoside triphosphates, and just prior to the pH break point (25 to 35 h) the concentration of all the nucleoside triphosphates decreased to almost zero (27 h). The nucleotide levels then increased rapidly as the morphology of the culture changed from phase-dark rods to phase-bright clostridial cells. The highest pool values for all the nucleoside triphosphates coincided with the peak in the

number of clostridial stage cells and the onset of forespore septum development, indicating that the nucleoside triphosphate pool values were higher in the stationary phase clostridial stage cells than in the exponential phase rods. The decrease in the number of clostridial cells after 47 h correlated with a decrease in the nucleoside triphosphate concentrations, maximum solvent levels and the initiation of spore maturation.

The nucleoside triphosphate profile for the *spo-1* mutant (Fig. 3.2b) was similar to that for the wild type P262 strain. ATP was the most abundant nucleoside triphosphate in exponential phase cells, followed by GTP, UTP and CTP. The *spo-1* mutant did not produce forespore septa, but the cells did produce a clostridial stage. The concentrations of all nucleoside triphosphates increased as the percentage of clostridial cells increased, and the peak concentrations coincided with the peak in the percentage of clostridial stage cells. In contrast to the wild type strain, the decrease in the nucleoside triphosphate levels after 50 h was not associated with a decrease in the percentage of clostridial stage cells. However, the metabolism of the *spo-1* mutant culture slowed down as solvent levels reached their maximum and the glucose concentration decreased.

The *cls-1* mutant was unable to produce solvents or to sporulate and did not differentiate into clostridial stage cells. The nucleotide levels in exponential phase cells of this mutant (Fig. 3.2c) were comparable to those observed in the wild type strain and the *spo-1* mutant. ATP was the most abundant nucleoside triphosphate, followed by GTP, UTP and CTP. The nucleoside triphosphate levels decreased as the cell number increased and acids were produced, reaching almost zero by 35 h. At this time, the cells had stopped dividing and the rate of acid production and glucose consumption had decreased. In contrast to the nucleoside triphosphate profiles obtained with the wild type strain and the *spo-1* mutant, the nucleoside triphosphate levels did not increase during the stationary phase, and after 40 h UTP and CTP were not detected.

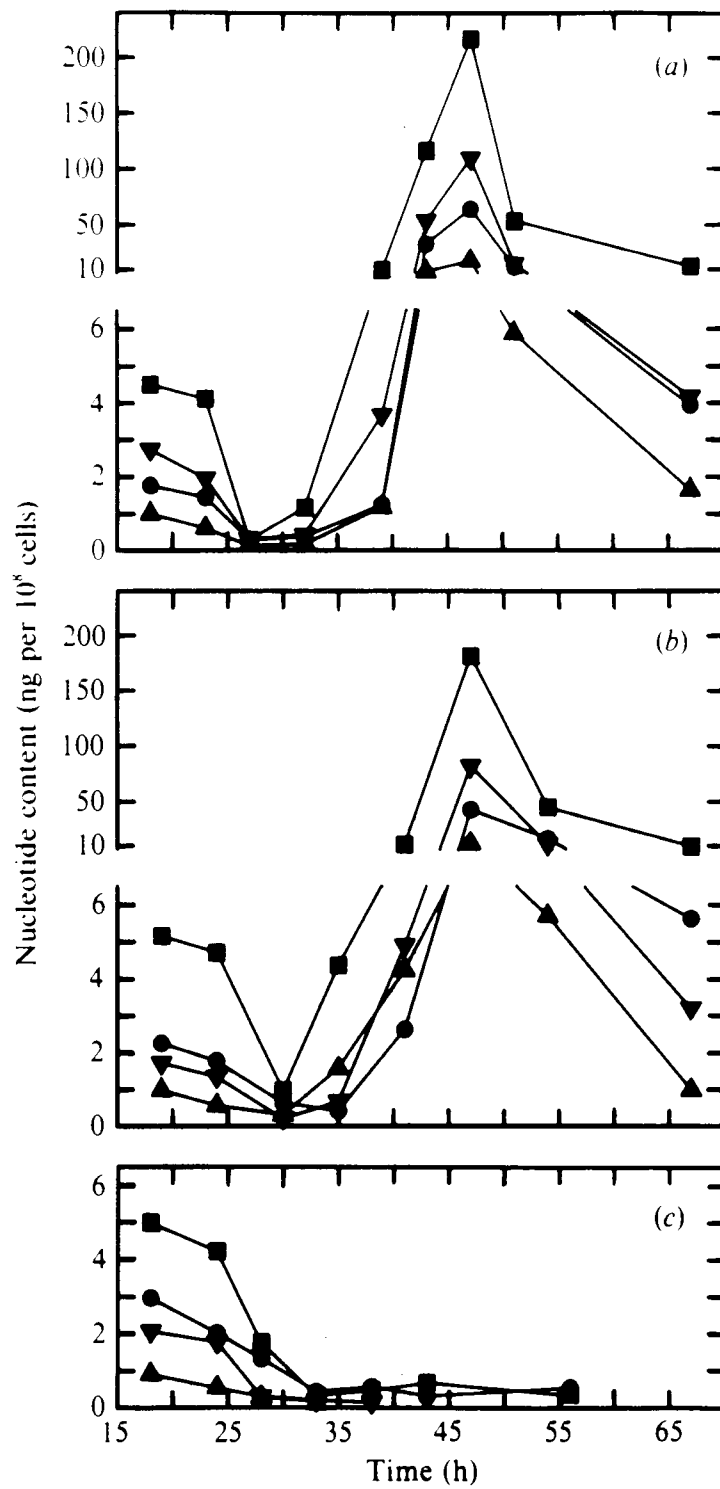


Figure 3.2 Nucleoside triphosphate profiles for *C. acetobutylicum*: (a) wild type strain P262, (b) the *spo-1* mutant, and (c) the *cls-1* mutant. In all plots the symbols correspond to the following: ■, ATP; ●, GTP; ▲, CTP; ▼, UTP.

Table 3.1. Percentage sporulation of wild type *C. acetobutylicum* P262 with and without the addition of compounds known to alter nucleotide levels.

Compound	Optimum Concentration ^a ($\mu\text{g ml}^{-1}$)	% Sporulation	
		Without Addition	With Addition
Cordycepin	300	33	42
Decoyinine	100	31	44
Psicofuranine	400	35	53
2-Aminomercaptopurine	500	39	60
6-Mercaptopurine	400	31	51
Caffeine	500	31	60
3-Methylxanthine	500	35	60
Theobromine	200	32	63
Theophylline	500	36	57
6-Azathymine	100	36	56

^a The optimum concentration of these compounds is the concentration that gave the greatest increase in percentage sporulation.

3.3.3 Nucleotide synthesis inhibitors

Ten compounds which increased the percentage of sporulating cells are shown in Table 3.1. Cordycepin, decoyinine and psicofuranine are adenosine analogues altered in the ribose moiety (Zain-ul-Abedin et al., 1983). Psicofuranine and decoyinine, which both inhibit GMP synthetase (Freese et al., 1979), were more effective than cordycepin at increasing the percentage of sporulating cells. The mercaptopurines (6-mercaptopurine and 2-aminomercaptopurine) were the next most effective compounds. The methylxanthines caffeine and theobromine provoked the largest increase in percentage sporulation, whereas the other two methylxanthines tested, theophylline and 3-methylxanthine, increased sporulation to a lesser extent. The pyrimidine analogue 6-azathymine also increased the percentage of sporulating cells.

3.4 Discussion

Nutrient shift-down experiments, in which rapidly dividing cells from a rich medium are transferred to a nutrient depleted medium, have been used to

induce sporulation in *Bacillus* species (Sterlini and Mandelstam, 1969). However, nutrient depletion does not induce sporulation in *C. acetobutylicum* P262, which sporulates only under conditions where growth is limited in the presence of excess glucose and ammonia (Long et al., 1984a). These studies were therefore carried out in the minimal medium CAMM, of Long et al. (1983), which provides a sufficiently synchronous developmental cycle to allow for correlative morphological, physiological and biochemical studies (Woods and Jones, 1986).

Nucleotides were quantitatively extracted from *C. acetobutylicum* cells by 3 M formic acid saturated with 1-butanol. The nucleotide extraction method of Olempska-Beer and Freese (1984) utilized 1 M formic acid saturated with 1-butanol. The concentration of formic acid had to be increased to 3 M to overcome the high buffering capacity of CAMM which contained calcium carbonate. This was consistent with the results of Bagnara and Finch (1972) who reported that formic acid was unsatisfactory for the extraction of nucleotides if the buffering capacity of the culture medium caused the pH of the extractant to rise above approximately pH 3. The formic acid extract had to be purified prior to HPLC analysis. Similar purification procedures were also reported as an essential step for the analysis of nucleotides from plant tissues (Meyer and Wagner, 1985) and from bacterial cells (Payne and Ames, 1982).

ATP was the most abundant nucleoside triphosphate produced by the wild type strain and the *spo-1* and *cls-1* mutants. An interesting observation was the extremely high concentrations of nucleotides in non-growing clostridial stage cells of the wild type strain and the *spo-1* mutant, since the fermentation of glucose to solvents yields 2 mol ATP per mol glucose, as compared to approximately 3.3 mol ATP per mol glucose when acids are produced (Jones and Woods, 1986a). However, the clostridial cells do not appear to elongate or divide and the nucleotides may accumulate as a result of the absence of growth. The high pool values of these energy rich compounds may explain the association of energy consuming processes such as granulose formation and capsule production with the clostridial stage (Reysenbach et al., 1986).

The decrease in ATP levels during the acidogenic phase could also be a result of rapid ATP consumption for maintenance of a constant ΔpH by proton extrusion. Acid production results in a decrease in the pH of the medium, and the cell must maintain a more or less constant ΔpH at the expense of ATP (Gottwald and Gottschalk, 1985; Huang et al., 1985). These results agree with the expected, in that the lowest ATP levels are associated with the pH breakpoint (Grupe and Gottschalk, 1990). The rate of proton extrusion, and hence the rate of ATP consumption, would be highest at the pH breakpoint where the external pH is at a low. The switch to solventogenesis and the reassimilation of acetic and butyric acids results in a rise in the pH of the medium. The maintenance of a ΔpH would therefore require less ATP and the ATP levels would begin to rise. Interestingly, in the case of the *cls-1* mutant, which was unable to switch to solventogenesis, ATP levels never rose.

The units that been used for reporting nucleoside triphosphate levels in *B. subtilis* are pmol per OD₆₀₀ unit (Ochi and Freese, 1983; Zain-ul-Abedin et al., 1983) or pmol per AM unit (Freese et al., 1981), where one AM unit is the amount of cells that produces an OD₆₀₀ of 1 if suspended in 1 ml. Unfortunately, these authors make no reference to cell number, nor do they mention the path length used in the optical density measurements. Therefore, the results presented here, which are based on cell number, are not directly comparable to those above which are based on optical density. With the *C. acetobutylicum* P262 strain, 10^8 cells ml⁻¹ gives an OD₆₀₀ of approximately 1 if a 1 cm path length is used. Therefore if the maximum nucleoside triphosphate concentrations reported by Zain-ul-Abedin et al. (1983) are converted from pmol per OD₆₀₀ unit to ng per OD₆₀₀ unit, the values obtained are approximately 375, 115, 91 and 41 for ATP, GTP, UTP and CTP respectively. Therefore, the nucleoside triphosphate levels for 1×10^8 *C. acetobutylicum* cells fall within the same range as reported for 1 OD₆₀₀ unit of *B. subtilis* cells.

The isolation of various classes and combinations of sporulation, clostridial cell, solvent, granulose and capsule mutants demonstrates that although the individual pathways function independently of each other, the induction of these

events appears to be linked by some common regulatory mechanism (Woods and Jones, 1986). The *spo-1* and *cls-1* mutants are examples of independent pathway and common regulatory mutants respectively. The concentrations of nucleoside triphosphates in the wild type strain and in the *cls-1* and *spo-1* mutants decreased during the late exponential growth phase and reached their lowest levels just prior to the pH break point. This trough also coincided with a decrease in motility and rate of cell division. In the wild type strain and the *spo-1* mutant the conversion of cells to the non-dividing clostridial stage and the onset of solvent production coincided with a rapid and very large increase in nucleotide pools. In the *cls-1* mutant, which failed to produce solvents or the clostridial stage, no increase in the level of nucleoside triphosphates was detected. The accumulation of high concentrations of nucleoside triphosphates in the *spo-1* mutant suggests that the increase in these compounds was associated with the conversion of cells to the solvent producing clostridial stage and was not dependent on spore formation.

A decrease in GTP has been shown to be associated with the initiation of differentiation and sporulation in *B. subtilis* (Lopez et al., 1979; Freese, 1981; Ochi and Freese, 1983), *Saccharomyces cerevisiae* (Freese et al., 1984b), and a streptomycete (Ochi, 1986). A variety of inhibitors which are known to decrease ribonucleotide levels in *B. subtilis* bring about an increase in sporulation (Freese et al., 1979; Ochi and Freese, 1983; Zain-ul-Abedin et al., 1983). Although there is no direct evidence implicating the low levels of GTP which occur during the late exponential growth phase in *C. acetobutylicum* P262 it is possible that a decrease in this or other nucleoside triphosphates plays a role in differentiation of *C. acetobutylicum*. The involvement of these compounds is supported by the increase in sporulation which was obtained in cultures treated with the various inhibitors shown in Table 3.1. When these inhibitors were added to stationary phase cultures just after the pH break point, little or no increase in sporulation occurred. This observation supports the suggestion of a common regulatory link between the shift to the clostridial stage and endospore formation. The methylxanthines were the most effective compounds in stimulating sporulation, which is consistent with the results of Sacks and Thompson (1977). One inhibitor

which would not be expected to affect purine ribonucleotide levels is the pyrimidine analogue 6-azathymine. This compound also caused an increase in sporulation in *C. acetobutylicum*. Similar results were obtained by Craven and Blankenship (1982) with *C. perfringens*. It can be speculated that a cascade of events must occur in order for *C. acetobutylicum* to sporulate. The initial trigger in the cascade would therefore take place at the switch from acidogenesis to solventogenesis. Although it is not direct evidence, it is interesting to note that a sporulating clostridial stage mutant has never been reported.

Chapter 4

Metronidazole activation and *E. coli*

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Chapter 4

Metronidazole activation and *E. coli*

4.0 Summary

E. coli strains with lesions in their DNA repair systems were more susceptible to metronidazole than wild type strains. The effect was more severe in *recA* mutants than in *uvr* mutants. The sensitivity of *E. coli* to metronidazole was tested under aerobic, anaerobic respiration and fermentation conditions. Anaerobically grown cultures were more sensitive to metronidazole than aerobically grown cultures, which were killed only when exposed to high levels of metronidazole. The lower redox potential of *E. coli* cells grown under fermentation conditions rendered them slightly more susceptible to metronidazole than anaerobically respiring cells. No synergistic effect between ampicillin and metronidazole was observed when testing the susceptibility of *E. coli* strains harbouring plasmid vectors which contained the β -lactamase gene. To isolate a suitable *E. coli* cloning host for the selection of *C. acetobutylicum* electron transport genes which activated metronidazole, transposon mutagenesis of the *recA* *E. coli* strain CC118 with *TnphoA*, was used to construct a *recA*, metronidazole resistant mutant (F19). F19 was shown to have diminished nitroreductase activity, which was presumed to be responsible for the metronidazole resistant phenotype. The *recA* mutation renders *E. coli* F19 highly susceptible to the reduced toxic intermediates of metronidazole. Thus, selecting for increased sensitivity to metronidazole makes F19 a suitable cloning host for the isolation of *C. acetobutylicum* electron transport genes which activate metronidazole, in *E. coli*.

4.1 Introduction

Although the spectrum of activity of metronidazole transcends many taxonomic boundaries, initial reports suggested that its toxicity was mainly limited to strict anaerobes and microaerophiles (Edwards, 1980), and that it was inactive against aerobes and facultative anaerobes except in concentrations that far exceeded those therapeutically attainable (Prince et al., 1969; Benazet et al., 1970; Ralph and Clarke, 1978; Onderdonk et al., 1979). Prince et al. (1969) tested metronidazole on a group of facultative anaerobes, and concluded that it had little or no clinical use for treatment of infections caused by these organisms. These authors did report that *E. coli* was slightly more sensitive than the other organisms tested, but the antibiotic concentration necessary for bacteriocidal action was still well above the obtainable serum concentrations at the recommended dosage. Edwards et al. (1979) reported a low incidence of clinical failure with metronidazole when treating patients for *Trichomonas vaginalis* infections. Swabs from these refractory patients yielded mixed cultures which typically contained *Streptococcus faecalis* and *E. coli*. The theory proposed for the clinical failures was that organisms other than *T. vaginalis* were able to absorb and bind metronidazole and hence decrease the concentration of the drug at the site of infection.

A comprehensive study by Onderdonk et al. (1979) was carried out where the pelvic cavity of Wistar rats was injected with either *E. coli* or a mixture of *E. coli* and *Bacteroides fragilis*. The rats that did not receive metronidazole all died due to *E. coli* septicemia, regardless of whether they were injected with *E. coli* or *E. coli* and *B. fragilis*. When the rats were treated with metronidazole, 2 out of 10 (20%) with mixed infections died, whereas 17 out of 20 (85%) with *E. coli* infections died. These authors carried this study one step further and analyzed the effect of metronidazole on continuous cultures of *E. coli* or *E. coli* and *B. fragilis*. In the case of *E. coli* alone, metronidazole was not being reduced and was therefore not biologically active. In the mixed culture, *B. fragilis* rapidly disappeared when the calculated concentration of metronidazole reached $1\text{ }\mu\text{g ml}^{-1}$. Concomitant with this was a temporary reduction in the viable

number of *E. coli*. The *E. coli* concentration returned to pre-treatment levels within 24 h. These authors concluded that *B. fragilis* reduced metronidazole to its active intermediate, and this intermediate escaped from the *B. fragilis* cells, and then entered and killed the *E. coli* cells. These results were supported in a later study by Soriano et al. (1982) where the antimicrobial activity of metronidazole was also measured against pure and mixed cultures of *E. coli* and *B. fragilis*.

Chrystal et al. (1980) demonstrated that the presence of *E. coli* enhanced the bacteriocidal effect of metronidazole against *B. fragilis*. They used a control culture where *B. fragilis* was inoculated into centrifuged and filtered medium that had supported the growth of *E. coli*. These authors also proposed that the reduced active intermediates of metronidazole were stable enough to diffuse from one cell to another. Hence they concluded that *E. coli* reacted with metronidazole to form a more active derivative which then diffused into the *B. fragilis* cells resulting in death. Their belief was that *B. fragilis* was more sensitive than *E. coli* to this particularly active derivative of metronidazole. Therefore, the presence of *E. coli* increased the concentration of this active derivative and this was responsible for the enhanced activity against *B. fragilis*.

Ingham et al. (1980) tested the effect of metronidazole on anaerobically grown cultures of *E. coli*. These authors showed that in the presence of $10\ \mu\text{g ml}^{-1}$ of metronidazole, there was an initial increase in the viable counts of *E. coli* cultures to 5×10^7 CFU ml^{-1} during the first 4 h. Thereafter the viable count dropped and eventually reached 4×10^4 CFU ml^{-1} after 24 h. This effect was faster and greater at a metronidazole concentration of $100\ \mu\text{g ml}^{-1}$, and was independent of inoculum size. In control cultures that were not treated with metronidazole, stationary phase was reached and the viable cell count after 24 h was 1×10^8 CFU ml^{-1} . Eight other strains of *E. coli*, 3 strains of *Klebsiella* and 6 out of 7 strains of *Proteus* gave almost identical results. Metronidazole had no effect on 6 strains of *Staphylococcus aureus* or 5 strains of *S. faecalis*. These authors refute the work of Onderdonk et al. (1979), and state that the presence of *B. fragilis* causes a decrease in the oxidation reduction potential of the environment, and hence metronidazole is more active against *E. coli*. They conclude that the killing of

facultative anaerobes does not require prior reduction of metronidazole, but merely an appropriately low redox environment. In a subsequent report (Ingham et al., 1981) these authors challenged the work of Chrystal et al. (1980). They prepared mixtures of *B. fragilis* or *B. fragilis* plus viable or heat killed *E. coli*. They also inoculated *B. fragilis* into centrifuged and filtered medium that had supported the growth of *E. coli*. In all cases they measured the redox potential (Eh), pH and dissolved oxygen concentration (pO_2). They confirmed that the rate of killing of *B. fragilis* was enhanced in the presence of viable or heat killed *E. coli* when compared to the control culture where *B. fragilis* was inoculated into medium that had previously supported the growth of *E. coli*. However, when compared to a control culture where *B. fragilis* was inoculated into freshly prepared medium, this enhanced rate of killing was not observed. Oxygen concentration measurements and redox measurements showed that the viable *E. coli* medium, the heat treated *E. coli* medium and the freshly prepared medium all had low pO_2 and Eh values. The centrifuged and filtered medium however had greater pO_2 and Eh values. They concluded that centrifugation and filtration introduced oxygen into the medium, which resulted in a slower killing rate of *B. fragilis* by metronidazole.

Moran et al. (1987) reported a 68% decrease in the viable cell count of *E. coli* cultures when treated with $10 \mu\text{g ml}^{-1}$ of metronidazole and a 99% decrease when treated with $100 \mu\text{g ml}^{-1}$ of metronidazole. They also showed that there was enhanced metronidazole sensitivity of both *E. coli* and *B. fragilis* when grown in mixed culture. It was suggested that this enhanced lethal effect against *B. fragilis* was due to a more reduced environment, and the enhanced lethal effect on *E. coli* was due to the presence of reduced intermediates excreted by *B. fragilis*. In a letter, Ingham and Sisson (1988) point out that these workers failed to employ stringent conditions of anaerobiosis, and that their conclusion that *B. fragilis* excreted metabolites of metronidazole was unjustified. The implication being that under strict anaerobic conditions, metronidazole was being reduced by *E. coli*.

The bacteriocidal toxic derivatives of metronidazole have been shown to cause DNA damage (Knight et al., 1978). Chrystal et al. (1980) proposed that reduced metronidazole can interact with either DNA to exert the bacteriocidal effect, or with water to form acetamide. Measuring metronidazole sensitivities of various *E. coli* strains which carried one or more genetic lesions in their DNA repair systems, Jackson et al. (1984) confirmed that DNA was the target molecule in this organism. The wild type strains were the least susceptible to metronidazole, whereas lesions in DNA repair genes conferred greater sensitivity to metronidazole. In all *E. coli* strains tested, metronidazole disappeared from the culture medium indicating that *E. coli* has the capacity to reduce metronidazole. In a similar study, Yeung et al. (1984) also tested the bacteriocidal activity of metronidazole against DNA repair mutant strains of *E. coli*. These DNA repair mutants of *E. coli* were killed more efficiently by metronidazole than their respective parental strains. However, if these DNA repair mutants also lacked the ability to reduce nitrate and chlorate, they were no more susceptible to metronidazole than their wild type parents. It was shown that metronidazole disappeared from the culture medium more slowly in the these nitroreductase deficient strains. Since acetamide concentration is proportional to the concentration of the active derivatives of metronidazole, these authors expressed metronidazole sensitivity in terms of acetamide concentration. This demonstrated that all of the strains with lesions in DNA repair genes were more susceptible to the reduced active derivatives of metronidazole than the wild type strains, whether or not they had diminished nitroreductase activity. This indicates that in *E. coli*, nitroreductase activity is obligatory for the activation of metronidazole, but not for the DNA damaging activity of the reduced derivatives. There are therefore two mechanisms of resistance to metronidazole, a decrease in the rate of formation of the reduced active derivatives, or an increased resistance at the target molecule, DNA.

In the absence of oxygen, *E. coli* cells breakdown pyruvate to produce formate and acetyl CoA by the action of the pyruvate formate lyase enzyme. Formate can be metabolized via the fermentative formate degradation pathway or via the

respiratory nitrate linked route (reviewed in Stewart, 1988). In the presence of a terminal electron acceptor such as nitrate, reduction is coupled to NADH oxidation to generate a proton motive force for membrane energization and hence energy production. In the fermentative pathway, formate is converted to carbon dioxide and hydrogen by the formate hydrogenlyase system (Peck and Gest, 1957). Formate hydrogenlyase consists of formate dehydrogenase, unknown electron carriers and hydrogenase (Stewart, 1988). The syntheses of the formate dehydrogenase and hydrogenase components of the formate hydrogenlyase pathway in *E. coli* are known to be repressed under aerobic conditions and anaerobically by nitrate (Wimpenny and Cole, 1967).

Two types of formate dehydrogenase activities have been identified in *E. coli*, and these may be distinguished by their activities with artificial electron acceptors (Stewart, 1988). Formate dehydrogenase-N (FDH_N) is present in nitrate respiring cells and is active with the artificial electron acceptors methylene blue, phenazine methosulfate, and dichlorophenolindolphenol. In contrast, the formate dehydrogenase-H (FDH_H), which is a component of the fermentative formate hydrogenlyase pathway, is active with methyl viologen and benzyl viologen as artificial electron acceptors. It can be inferred from the oxidation reduction potential of these artificial electron acceptors, that the redox environment is lower under fermentation conditions than under conditions of anaerobic respiration.

Since the redox environment has been shown to play a crucial role in metronidazole susceptibility (Ingham et al., 1981), it is imperative to determine, in the case of facultative anaerobes, if metabolism is occurring via anaerobic respiration or fermentation. The purpose of this study was to determine the effect of metronidazole on various strains of *E. coli*, under conditions of fermentation and anaerobic respiration with the view to creating a suitable system for the cloning of *C. acetobutylicum* electron transport genes that may activate metronidazole.

4.2 Materials and Methods

Commonly used materials and techniques are presented in the Appendices. The details provided below are included for completeness.

4.2.1 Bacterial strains

The bacterial strains used in this study are shown in Table 4.1, and are described in Appendix C. The nitroreductase deficient *E. coli* strain F19 was derived from the *recA E. coli* strain CC118 (*araD139Δ(ara, leu)7697ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argEam recA1*) (Manoil and Beckwith, 1985) by transposon mutagenesis. Phage λ b221*rex::TnphoA* cI857 Pam3 was a gift from C. Manoil (Gutierrez et al., 1987).

4.2.2 Media and culture conditions

E. coli was grown aerobically in Luria-Bertani (LB) medium. Anaerobic growth of *E. coli* was carried out in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio). The medium used for growth of *E. coli* under conditions of anaerobic respiration was LB medium containing 0.1% glucose and 0.5% sodium nitrate (LBR). The medium used for fermentative growth of *E. coli* was either LB medium containing 1.0% glucose (LBF) or the complex medium (pH 8.0) described by Clark and Cronan (1980) supplemented with 1% glucose (ECAM). When necessary, 1.5% agar was added to these media.

4.2.3 Determination of MIC

An anaerobically grown overnight liquid culture of *E. coli* was diluted 10^{-4} with anaerobic sterile water, and 100 μ l was spread onto pre-reduced agar medium containing various concentrations of metronidazole. The MIC was taken as the amount of antibiotic (per ml) that totally inhibited growth after incubation for 24 h at 34°C.

4.2.4 Transposon mutagenesis

TnphoA is a derivative of Tn5 which contains the structural gene for *E. coli* alkaline phosphatase (*phoA*) cloned into the transposon (Manoil and Beckwith, 1985). The alkaline phosphatase portion of *TnphoA* was not needed for the desired *E. coli* chromosomal mutation, and hence *TnphoA* was merely used in

preference to Tn5 because it was readily available. Transposon insertions of Tn*phoA* into *E. coli* (CC118) were obtained using an adaptation of the protocol of Gutierrez et al. (1987). A sample (1 ml) of an overnight culture of *E. coli* CC118 in LB containing 10 mM magnesium sulfate was mixed with phage λ ::Tn*phoA* at a multiplicity of infection of approximately 1, and was incubated at 30°C for 4 h to allow for outgrowth of the phage. Samples (100 μ l each) were plated onto LB plates containing 30 μ g ml⁻¹ of kanamycin and incubated overnight at 30°C. Colonies from each plate were pooled into 1 ml of sterile water. From each cell/water pool, 100 μ l was plated onto LBR medium containing 30 μ g ml⁻¹ of kanamycin and 30 μ g ml⁻¹ metronidazole and incubated anaerobically at 34°C. Metronidazole resistant mutants were tested for *recA* reversion and nitroreductase activity.

4.2.5 Nitroreductase assay

To test the ability of *E. coli* to reduce nitro-heterocyclic compounds, the conversion of *p*-nitrobenzoic acid (PNBA) to *p*-aminobenzoic acid (PABA) was monitored. PABA concentration was measured using an assay procedure originally developed for sulfanilamide determination (Bratton and Marshall, 1939). One ml of an anaerobically grown overnight culture of *E. coli* was added to 5 ml of pre-reduced LB supplemented with 120 μ g ml⁻¹ of PNBA (final concentration of PNBA = 100 μ g ml⁻¹). A 100 μ l sample was removed immediately and assayed for PABA (time 0), and the remainder was incubated anaerobically at 34°C. Samples were then assayed for PABA at hourly intervals for six hours. To assay for PABA, the 100 μ l sample was placed in a test tube containing 2.3 ml of water, 600 μ l of 15% tri-chloroacetic acid, and 300 μ l of 0.1% sodium nitrite. After standing for at least 3 min, 300 μ l of 0.5% ammonium sulfamate was added to the tube. After standing for another 3 min, 300 μ l of 0.1% N-(1-naphthyl)ethylene diamine dihydrochloride was added to the tube. The absorbance was measured at 540 nm against a blank of LB containing 100 μ g ml⁻¹ PNBA, which was similarly treated. The PABA concentration was calculated using the least squares regression line equation determined by assaying known concentrations of PABA.

Table 4.1. The relationship between DNA repair mutations and metronidazole sensitivity of *E. coli* strains.

Strain	MIC ^a	DNA repair mutation	Reference
C600	150	-	Wood, 1966
JM103	150	-	Yanisch-Perron et al., 1985
CSH50	150	-	Miller, 1972
YMC11	150	-	Backman et al., 1981
K514	150	-	Zabeau & Stanley, 1982
ET8051	150	-	Pahel & Tyler, 1979
AB1157	150	-	DeWit & Adelberg, 1962
AB1886	35	<i>uvrA</i>	Howard-Flanders et al., 1966
AB1885	35	<i>uvrB</i>	Howard-Flanders et al., 1966
AB1884	35	<i>uvrC</i>	Howard-Flanders et al., 1966
N14-4	40	<i>uvrD</i>	Ogawa et al., 1968
HB101	20	<i>recA</i>	Boyer & Roulland-Dussoix, 1969
JA221	20	<i>recA</i>	Beggs, 1978
CC118	20	<i>recA</i>	Manoil & Beckwith, 1985
LK111	15	<i>recA</i>	Zabeau & Stanley, 1982
CSR603	15	<i>recA, uvrA</i>	Sancar & Rupert, 1978
F19	35	<i>recA</i>	this study

^a All MIC values are in $\mu\text{g ml}^{-1}$. Media used for MIC determinations was LB supplemented with 0.5% (w/v) NaNO_3 and 0.1% (w/v) glucose which was incubated anaerobically at 34°C.

4.2.6 Testing for *RecA* phenotype

The standard UV dose experiment described by Maniatis et al. (1982) was used to test the stability of DNA repair mutations. A fluence of $1 \text{ J m}^{-2} \text{ sec}^{-1}$ was used to irradiate single streaks of the various *E. coli* strains. *RecA* mutants were unable to grow after a 5 sec exposure, whereas wild type strains survived a 25 sec exposure.

4.3 Results

4.3.1 DNA repair mutations and metronidazole sensitivity

E. coli strains containing various DNA repair mutations were tested for their susceptibility to metronidazole (Table 4.1). Strains with a complete and functional DNA repair system were not as sensitive to metronidazole as strains

Table 4.2 Metronidazole sensitivity of *E. coli* strains grown under aerobic (LB), anaerobic respiration (LBR) and fermentation (LBF and ECAM) conditions.

Medium ^a (Incubation)	<i>E. coli</i> Strain (Plasmid)	MIC ($\mu\text{g ml}^{-1}$)
LBR (anaerobic)	CC118	20
	CC118 [pBR322]	20
	F19	35
	F19 [pBR322]	35
	C600	150
	C600 [pBR322]	150
LBF (anaerobic)	CC118	6
	CC118 [pBR322]	6
	F19	15
	F19 [pBR322]	15
	C600	110
	C600 [pBR322]	110
ECAM (anaerobic)	CC118	6
	CC118 [pBR322]	6
	F19	15
	F19 [pBR322]	15
	C600	110
	C600 [pBR322]	110
LB (aerobic)	CC118	100
	CC118 [pBR322]	100
	F19	200
	F19 [pBR322]	200
	C600	1500
	C600 [pBR322]	1500

^a Media used to support growth of *E. coli* cells which contained plasmids was supplemented with $100 \mu\text{g ml}^{-1}$ of ampicillin.

with mutations in their DNA repair systems. Strains with *uvr* mutations were more sensitive to metronidazole than wild type strains, and strains with *recA* mutations were even more sensitive than strains with *uvr* mutations.

4.3.2 Growth conditions and metronidazole sensitivity

E. coli strains grown under aerobic, anaerobic respiration and fermentation conditions were tested for their susceptibility to metronidazole (Table 4.2). All

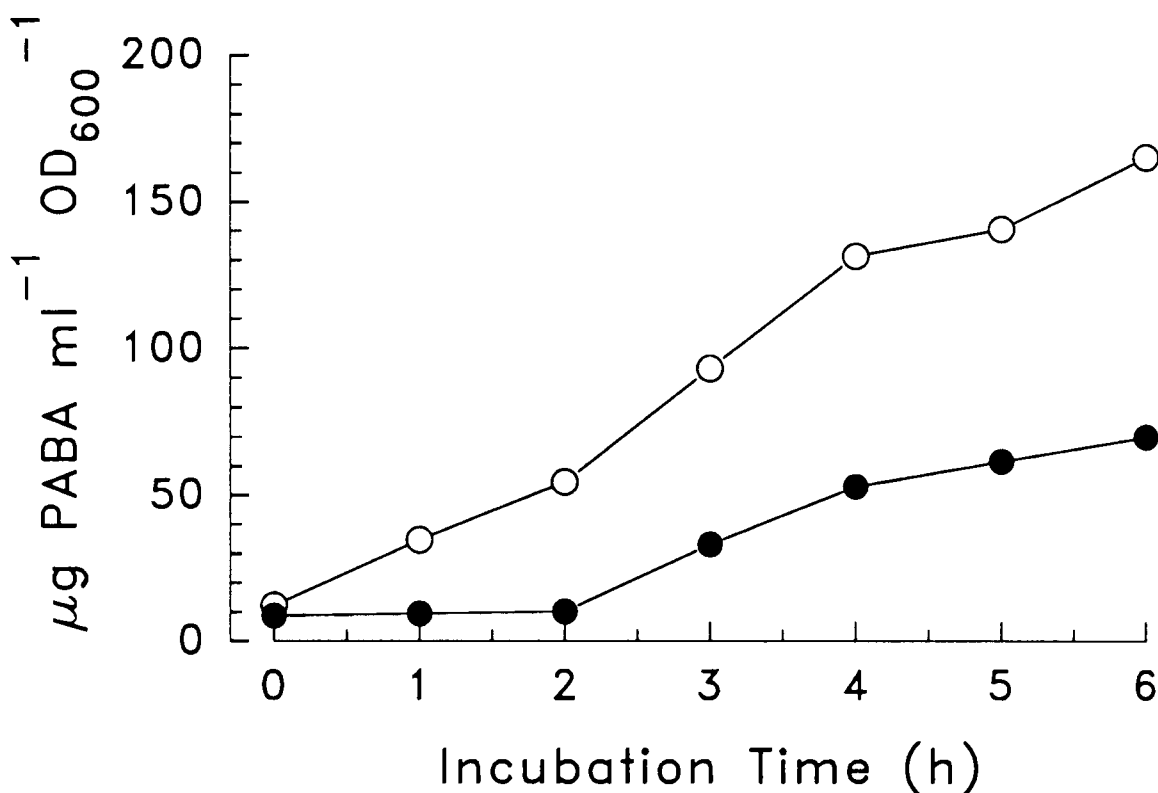


Figure 4.1 Nitroreductase activity of *E. coli* F19 (●) as compared to its parent, *E. coli* CC118 (○). Nitroreductase activity was determined by incubating cells with *p*-nitrobenzoic acid and measuring the resulting *p*-aminobenzoic acid (PABA) concentration with respect to time.

strains were more sensitive to metronidazole when grown under anaerobic conditions. *E. coli* strains grown under fermentation conditions were more sensitive to metronidazole as compared to strains grown under anaerobic respiration. Ampicillin ($100 \mu\text{g ml}^{-1}$) was added to media used to support the growth of *E. coli* strains containing the plasmid pBR322. No synergistic effect between metronidazole and ampicillin was demonstrated.

4.3.3 Transposon mutagenesis

E. coli CC118 was a suitable strain for transposon mutagenesis since it has a stable *recA* mutation, is not a λ -lysogen, is readily transformable and is a proven host for *TnphoA* (Manoil and Beckwith, 1985). Following transposon mutagenesis, 222 metronidazole resistant mutants were isolated.

4.3.4 Nitroreductase activities and *recA* stability

The 222 metronidazole resistant mutants isolated by transposon mutagenesis were tested for stability of mutation, *recA* reversion, and nitroreductase activity. Of the 222 mutants, 10 were not stable and were unable to grow in the presence of metronidazole after subculturing. A further 29 were *recA* revertants, while 50 did not show reduced nitroreductase activity. The remaining 133 mutants had a diminished ability to reduce nitroheterocyclic compounds. Mutant F19 consistently showed the lowest level of nitroreductase activity, and the nitroreductase activity of this mutant is compared to that of its parent CC118 in Fig. 4.1. Mutant F19 was therefore chosen to screen the *C. acetobutylicum* gene bank for genes which cause an increase in sensitivity towards metronidazole (see chapter 5).

4.4 Discussion

Chrystal et al. (1980) proposed a model relating metronidazole metabolism to bacteriocidal action, and a similar model is presented in Fig. 4.2. Metronidazole is reduced to its active form M^* , which can react with DNA resulting in DNA damage. If the rate of DNA damage (K_{ii}) is greater than the rate of repair (K_{iii}), cell death occurs. Therefore a cell with an efficient DNA repair mechanism is able to tolerate higher levels of M^* than a cell with a less efficient DNA repair system. Since the rate of formation of M^* (K_i) is proportional to the rate of DNA damage, two mechanisms of resistance to metronidazole exist. One mechanism involves an efficient DNA repair system and the other involves the lack of the ability to reduce metronidazole to its active intermediate M^* .

In agreement with Jackson et al. (1984) and Yeung et al. (1984), the results in Table 4.1 show that of all the *E. coli* strains tested, those with one or more lesions in their DNA repair systems were more susceptible, to varying degrees, than strains with complete and functional DNA repair system. An MIC of $40 \mu\text{g ml}^{-1}$ was observed for the *uvrD* mutant (N14-4), while the next susceptible strains were the *uvrA* (AB1886), *uvrB* (AB1885) and *uvrC* (AB1884) strains, which had an MIC of $35 \mu\text{g ml}^{-1}$. With the exception of strain LK111 which has an MIC of

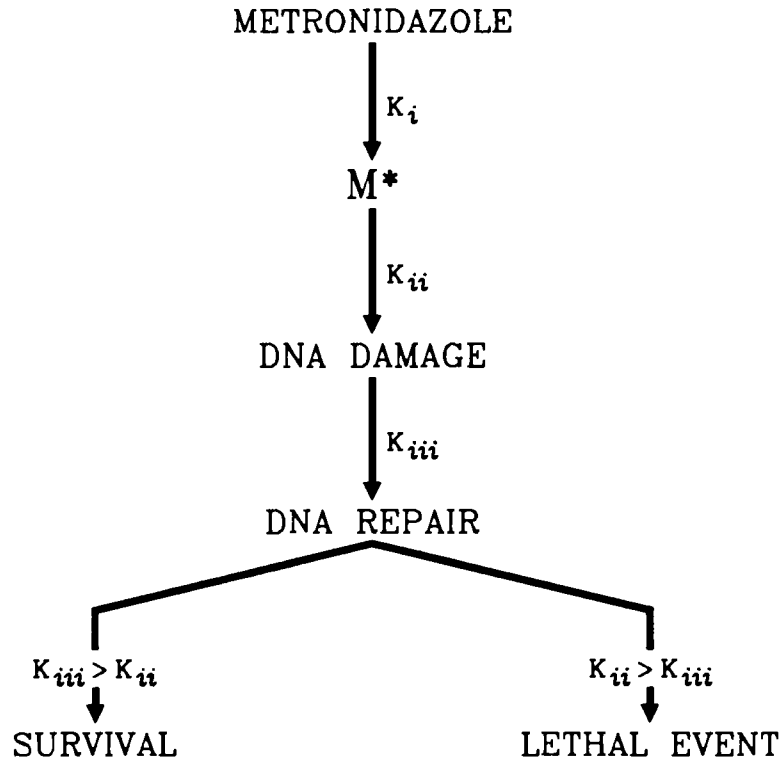


Figure 4.2 A model relating metabolism of metronidazole to cell death. Metronidazole is reduced to its active derivative M^* by the cell. M^* reacts with DNA causing damage, which can be repaired by the host cell's DNA repair systems. K_i is the rate of reduction of metronidazole, K_{ii} is the rate of DNA damage, and K_{iii} is the rate of DNA repair. If the rate of DNA damage (K_{ii}) is greater than the rate of DNA repair (K_{iii}), cell death occurs.

15 $\mu\text{g ml}^{-1}$, all of the *recA* only mutants had an MIC of 20 $\mu\text{g ml}^{-1}$. Therefore, with respect to single lesions in the DNA repair systems, the *recA* mutation proved to have the largest effect. As shown by the MIC of strain CSR603 (*recA*, *uvrA*), additional lesions in DNA repair systems caused an enhanced effect. Similar results were reported by Jackson et al. (1984) with *lexA*, *uvrA* and *recA*, *uvrA* double mutants. *E. coli* strain F19 was specifically developed to be resistant to metronidazole, as is discussed in more detail below.

Under aerobic conditions, *E. coli* cells respire which leads to the transfer of electrons to oxygen. Metronidazole is an artificial electron acceptor for low redox potential electrons (Müller, 1983). Therefore, metronidazole is in competition with oxygen for available electrons in aerobically grown cells. It is believed that the reduction of metronidazole occurs in single electron steps,

leading to the formation of a free radical anion (Perez-Reyes et al., 1979). In the presence of oxygen metronidazole can be reduced, albeit slowly, to the single electron transfer free radical anion, which is reoxidized by oxygen with the production of superoxide. It can be speculated that the superoxide radical, which is known to cause DNA damage, may be responsible for the bacteriocidal effect high concentrations of metronidazole have on aerobically grown *E. coli* cells (Table 4.2), and that metronidazole itself is not directly involved. Under anaerobic conditions, the cellular redox potential is related to the reduction of metronidazole to its active derivative or derivatives (beyond single electron transfer). As is shown in Table 4.2, anaerobically respiring *E. coli* cells are slightly less susceptible than fermenting cells, presumably due to the slightly less negative intracellular redox potential. The results in Table 4.2 also support the data in Table 4.1, where *E. coli* strains CC118 and F19, which are *recA* mutants, are more susceptible to metronidazole than the wild type C600 strain. This observation occurred even in aerobically grown cells, indicating that DNA damage is still the mechanism by which cell death occurs.

Ampicillin did not have a synergistic effect when used in conjunction with metronidazole. This is an important observation in lieu of the development of an *E. coli* cloning host for use with vectors containing a β -lactamase resistance marker. This desired cloning host should lack the ability to reduce metronidazole to its active intermediates, while being highly susceptible to these active compounds. As shown in Table 4.1, DNA repair deficient mutants of *E. coli* are more susceptible to metronidazole than their wild type parents. However, Yeung et al. (1984) demonstrated that nitrate reductase, DNA repair double mutants were no more susceptible to metronidazole than their wild type parents. Furthermore, these authors showed that these double mutants were equally sensitive to the active intermediates of metronidazole, when compared to other DNA repair mutants, but they reduced metronidazole at a slower rate. The latter feature was presumed to be due to the nitrate reductase mutation. Nitrate reductase mutants of *E. coli* are easily obtained by selecting for chlorate resistance (Begg et al., 1977; Graham et al., 1980; Stewart and McGregor, 1982). Since a primary goal of this study was to isolate an *E. coli* mutant with

diminished ability to reduce metronidazole, it was decided to select directly for metronidazole resistance as opposed to chlorate resistance. Furthermore, as metronidazole is not a nitrate compound per se, but rather a nitroheterocyclic compound, further screening of these metronidazole resistant mutants was carried out by measuring their ability to reduce PNBA to PABA. Transposon mutagenesis of *E. coli* CC118 yielded 133 stable metronidazole resistant, *recA* mutants. Mutant F19 consistently showed the lowest level of nitroreductase activity (Fig. 4.1). This mutant has the desired traits of a diminished ability to reduce metronidazole, presumably due to the low level of nitroreductase, while remaining highly sensitive to the reduced active derivatives of metronidazole, due to the *recA* mutation. F19 was therefore chosen as the cloning host to screen for recombinant plasmids containing *C. acetobutylicum* DNA coding for electron transport genes that activate metronidazole (see Chapter 5). Since this mutant was developed to be used as a cloning host, and not for physiological studies of the effects of metronidazole on *E. coli*, no efforts were made to further characterize the mutation induced by *TnphoA*.

Chapter 5

Isolation and classification of *C. acetobutylicum* genes which activate metronidazole

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Chapter 5

Isolation and classification of *C. acetobutylicum* genes which activate metronidazole

5.0 Summary

The *E. coli* F19 *recA*, nitroreductase deficient mutant (Chapter 4) was used for the isolation of *C. acetobutylicum* genes on recombinant plasmids which activated metronidazole. These genes rendered the *E. coli* F19 strain sensitive to metronidazole. Twenty five *E. coli* F19 clones which contained different recombinant plasmids were isolated and classified into five groups (I-V) based on their sensitivity to metronidazole. With respect to metronidazole susceptibility, class I isolates were the least sensitive group whereas class V isolates were the most sensitive group. The clones were tested for nitroreductase, pyruvate-Fd-oxidoreductase and hydrogenase activities. Nitroreductase and pyruvate-Fd-oxidoreductase activity was not demonstrated in any of the isolated clones. Only one clone pMET15B2, which is a class I isolate, tested positive for hydrogenase activity. DNA hybridization and restriction endonuclease mapping revealed that four of the *C. acetobutylicum* insert DNA fragments on recombinant plasmids were linked in an 11.1 kb chromosomal fragment. Three of these clones, pMET13A, pMET030 and pMET190, were class V isolates, whereas the fourth clone was the hydrogenase positive isolate pMET15B2. The 11.1 kb chromosomal fragment therefore contained at least two regions responsible for activating metronidazole. The region responsible for class V sensitivity to metronidazole was localized to a 2 kb *EcoRI-EcoRV* fragment, which was present in each of the clones pMET13A, pMET030 and pMET190. Based on the orientation of the insert DNA with respect to vector DNA it was concluded that the gene responsible for class V sensitivity to metronidazole located on this 2 kb region was transcribed from a promoter present on the insert.

5.1 Introduction

Based on the principal that electron flow can control carbon flow (Kim et al., 1984; Jones and Woods, 1986a), genes encoding electron transport proteins and enzymes may play an important role in genetic manipulations of *C. acetobutylicum* aimed at improving the fermentation. Furthermore, an understanding of the mechanisms involved in the activation of metronidazole is important for the development of improved analogs for use in medicine. To gain knowledge of both metronidazole activation and *C. acetobutylicum* electron transport genes, a negative selection system was developed for cloning *C. acetobutylicum* electron transport genes in *E. coli* that activate metronidazole (see also chapter 4). The cloning strategy involved the use of the nitroreductase deficient, *recA E. coli* F19 mutant. *C. acetobutylicum* genes on recombinant plasmids which activate metronidazole would complement this mutant's deficiency in reducing the drug. These genes can be identified by observing an increased sensitivity to metronidazole.

There have been conflicting reports as to what proteins or enzymes are responsible for the reduction of metronidazole to its active derivatives. Edwards and Mathison (1970) reported that reduced ferredoxin could directly transfer electrons to metronidazole, forming the reduced toxic derivatives. This theory is supported by the hydrogenase linked assay system developed by Chen and Blanchard (1979) for measuring ferredoxin and flavodoxin, using metronidazole as the indicator compound. These authors report that hydrogenase transfers electrons from hydrogen gas to ferredoxin or flavodoxin which then transfer electrons to metronidazole forming the reduced toxic intermediates. It has also been reported that metronidazole is reduced enzymatically by the action of pyruvate-Fd-oxidoreductase (Narikawa, 1986). The most recent theory has been put forward by Church et al (1988, 1990) who have shown that metronidazole as well as other nitroimidazole drugs are reduced by hydrogenase 1 of *C. pasteurianum*. Any of the clostridial genes encoding electron carriers or enzymes involved in the phosphoroclastic breakdown of pyruvate and the concomitant

production of hydrogen gas are therefore likely candidates to be isolated using this cloning strategy.

The work described in this chapter involved the screening of a *C. acetobutylicum* DNA fragment library in *E. coli* F19 for genes that activate metronidazole.

5.2 Materials and Methods

Commonly used materials and techniques are presented in the Appendices. The details provided below are included for completeness.

5.2.1 Chemicals

Restriction endonucleases were obtained from several sources (Amersham International, Anglian Biotec, Boehringer Mannheim Biochemicals GmbH, New England Biolabs, and the Promega Corp.) [α - 32 P] labelled dATP was obtained from Amersham International. Metronidazole was obtained from the Sigma Chemical Co. All other chemicals were analytical grade and were purchased from various local agents and suppliers.

5.2.2 Bacterial strains and plasmids

C. acetobutylicum P262 (Jones et al., 1982) was used as the source of DNA. *E. coli* strain F19 (Santangelo et al., 1991) was used as the recipient strain for recombinant plasmids. Construction of the nitroreductase deficient, *recA* *E. coli* strain F19 is described in Chapter 4. For hydrogenase assays, plasmids were transformed into the *E. coli* SE53 *hydA* mutant which was derived from *E. coli* strain Puig426 (Lee et al., 1985). The plasmid vector pEcoR251 (Appendix E) which was a gift from M. Zabeau, Biotechnology Business Development, Ghent, Belgium, was used to prepare a genomic library of *C. acetobutylicum* chromosomal DNA (Zappe et al., 1986). pEcoR251 is a positive selection vector containing the *E. coli* *EcoR1* gene under the control of the λ -rightward promoter, the pBR322 ampicillin resistance gene and the pBR322 origin of replication. The *EcoR1* gene product, when expressed at high levels by the λ promoter is lethal unless insertionally inactivated, or regulated by a resident λ prophage. The *EcoR1* gene has single restriction enzyme sites for *HindIII*, *BglII* and *SspI*. Positive

selection is obtained through insertional inactivation of the *EcoR*I endonuclease gene, by cloning foreign DNA into one of these single restriction enzyme sites. pEcoR251 was derived from the pCL series of plasmids described by Zabeau and Stanley (1982).

5.2.3 Media and growth conditions

C. acetobutylicum P262 was grown under strict anaerobic conditions in the *Clostridium* basal medium (CBM) of O'Brien and Morris (1971) as described by Allcock et al. (1982). *E. coli* F19 was grown as described in section 4.2.2.

5.2.4 Determination of MIC

MIC values were determined as described in section 4.2.3.

5.5.5 Preparation of DNA

Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowitz and Burke (1981). The small-scale (miniprep) and large-scale (maxiprep) methods of plasmid isolation are described in Appendix B. *C. acetobutylicum* cellular DNA was prepared by the method of Marmur (1961), which was modified (Zappe et al., 1986) to overcome the high nuclease activity exhibited by this organism (Urano et al., 1983). *E. coli* cellular DNA was prepared as described by Maniatis et al. (1982).

5.2.6 Genebank construction

The genomic library (Zappe et al., 1986) contained sucrose fractionated *Sau*3A1 endonuclease fragments (4 to 7 kb from a partial digestion) of *C. acetobutylicum* P262 chromosomal DNA which were ligated with pEcoR251 which had been restricted with *Bgl*II endonuclease.

5.2.7 Cloning of *C. acetobutylicum* genes which activate metronidazole

E. coli F19 cells containing recombinant pEcoR251 plasmids were selected on LB agar containing ampicillin (100 µg ml⁻¹). Colonies were duplicated onto LBR agar containing ampicillin (100 µg ml⁻¹) or ampicillin plus metronidazole (30 µg ml⁻¹). These plates were incubated anaerobically at 34°C for 24 h, and

colonies that were resistant to ampicillin and sensitive to metronidazole were chosen for further study.

5.2.8 Preparation of cell extracts

Cell extracts of *E. coli* were prepared from overnight cultures (400 ml) grown anaerobically in the presence of ampicillin ($100 \mu\text{g ml}^{-1}$). Cell extracts of *C. acetobutylicum* were prepared from exponential phase cells grown anaerobically in CBM. Cell extracts were prepared under anaerobic conditions by the procedure of Clark and Cronan (1980) and stored at -70°C to preserve enzyme activity. Protein concentrations in the extracts were determined by the biuret method as described by Gornall et al. (1949).

5.2.9 Enzyme assays

Nitroreductase activity was assayed by determining the conversion of PNBA to PABA according to the method of Bratton and Marshall (1939), as described in section 4.2.5.

Pyruvate-Fd-oxidoreductase was assayed using the method of Wahl and Orme-Johnson (1987). The reaction mix contained 10 mM sodium pyruvate, $250 \mu\text{M}$ 2-mercaptoethanol, $250 \mu\text{M}$ coenzyme A, $50 \mu\text{M}$ thiamine pyrophosphate and 2 mM methyl viologen in 50 mM Tris-HCl (pH 7.5). The reaction was initiated by the addition of the cell free extract ($500 \mu\text{g}$ protein). The reaction was monitored by following the absorbance at 600 nm. All manipulations and measurements were carried out under strict anaerobic conditions in an anaerobic cabinet.

Hydrogenase activity was determined by testing the ability of cell free extracts to produce hydrogen gas from the reduced artificial electron acceptor, methyl viologen (Church et al., 1988). Assays were performed using a Warburg apparatus with a water bath temperature of 30°C . Warburg flasks were gassed out for 20 min with helium. The centre well contained $200 \mu\text{l}$ of 20% potassium hydroxide to absorb any carbon dioxide which evolved during the reaction. The rim of the centre well was coated with a thin layer of vacuum grease to prevent

creeping of the potassium hydroxide. The reaction flask contained 10 mM methyl viologen and 15 mM sodium dithionite in 50 mM Tris-HCl (pH 7.5). The reaction was started by tipping a volume of the cell free extract equivalent to 3 mg of protein, into the main compartment of the reaction vessel. The final volume in the reaction flask was always 2 ml, and hydrogen production was monitored over 10 min. The volume of hydrogen produced was calculated according to the methods described by Umbreit et al. (1972).

5.2.10 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (PAGE) was carried out by following the methods of Laemmli (1970), using 50 µg per lane of proteins from crude cell extracts. The gels were electrophoresed at 35 mA constant current using a Hoeffer SE 600 vertical gel apparatus. The 1.5 mm thick gels consisted of 10% acrylamide with 2.7% crosslinking. After electrophoresis, proteins were stained with Coomassie blue R-250.

5.2.11 Restriction mapping

Recombinant plasmids were characterized by restriction mapping using standard procedures (Maniatis et al., 1982).

5.2.12 Preparation of radiolabelled probe DNA

Plasmids containing *C. acetobutylicum* DNA inserts, or isolated DNA fragments were nick translated (Rigby et al., 1977) with [α -³²P]dATP using the nick translation kit of Amersham International (code N.5500). Nick translated probes were prepared according to the manufacturer's instructions.

5.2.13 DNA hybridization

Plasmid DNA, *C. acetobutylicum* chromosomal DNA and *E. coli* chromosomal DNA were digested to completion with the appropriate endonucleases, and the resulting fragments were fractionated by electrophoresis in either 0.8% or 1.2% (w/v) agarose gels in Tris-acetate buffer, as appropriate. The DNA was transferred monodirectionally to a Hybond N⁺ nylon membrane (Amersham International) according to the manufacturer's instructions. Radiolabelled DNA,

described above, was used to probe these membranes (Southern, 1975; Smith and Summers, 1980).

5.3 Results

5.3.1 Screening of the *C. acetobutylicum* gene library

E. coli F19 was used as the host to screen the *C. acetobutylicum* gene library for genes conferring metronidazole sensitivity. Although this mutant was very sensitive towards the reduced toxic derivative(s) of metronidazole due to a *recA* mutation, it was resistant to metronidazole since it was deficient in nitroreductase activity associated with the reduction of metronidazole. The screening of the *C. acetobutylicum* gene library in *E. coli* F19 selected for genes that complement the metronidazole reducing ability which is lost in this mutant. Twenty six stable clones were isolated that showed an increased sensitivity towards metronidazole. Plasmid involvement in metronidazole susceptibility was confirmed by retransformation experiments with plasmids isolated from the 26 clones. Plasmids from each clone retransformed into *E. coli* F19 produced similar numbers of ampicillin resistant and metronidazole sensitive transformants.

5.3.2 Classification of clones based on MIC

The 26 metronidazole sensitive clones were classified into five groups (I-V) based on their susceptibility to metronidazole, where class V was the most sensitive group and class I was the least sensitive group (Table 5.1). All clones were more sensitive to metronidazole than the *E. coli* F19 mutant.

5.3.3 Tests for enzyme activity

Since it has been reported that clostridial enzymes, pyruvate-Fd-oxidoreductase (Narikawa, 1986) and hydrogenase (Church et al., 1988, 1990) may be responsible for the reduction of metronidazole, cell extracts of all 26 clones were tested for these enzymes. Since the enzyme activity responsible for the reduction of metronidazole in *E. coli* involves a nitroreductase (Yeung et al., 1984), the 26 clones were also tested for nitroreductase activity. No significant nitroreductase

Table 5.1 Metronidazole sensitivity of the twenty six *E. coli* clones containing recombinant plasmids with DNA inserts from *C. acetobutylicum* gene library. MIC's were determined under conditions of anaerobic respiration and fermentation.

Isolate ^a	MIC ($\mu\text{g ml}^{-1}$)		Sensitivity Class ^b
	respiration	fermentation	
F19 ^c	35	15	-
CC118 ^c	20	6	-
pMET11B	<5	<1	V
pMET13A	<5	<1	V
pMET13C1	<5	<1	V
pMET030	<5	<1	V
pMET190	<5	<1	V
pMET4B	10	3	IV
pMET5E	10	3	IV
pMET10B	10	3	IV
pMET9D	12	4	III
pMET10D	12	4	III
pMET14B	12	4	III
pMET2D	15	5	II
pMET4A	15	5	II
pMET8E	15	5	II
pMET1A	20	6	I
pMET7A	20	6	I
pMET7C	20	6	I
pMET9A	20	6	I
pMET13B	20	6	I
pMET13C2	20	6	I
pMET15B2	20	6	I
pMET15D	20	6	I
pMET020	20	6	I
pMET040	20	6	I
pMET130	20	6	I
pMET140	20	6	I

^a All isolates represent *C. acetobutylicum* DNA cloned into plasmid pEcoR251 which was transformed into *E. coli* F19.

^b Sensitivity class is a arbitrary numbering system based on the observed MIC. Class V is the most sensitive class towards metronidazole whereas Class I is the least sensitive class.

^c *E. coli* strains CC118 and F19 are included as controls for comparative purposes. Plasmid pBR322 was transformed into these control strains in order to determine the MIC on the same medium as the clones, which contained ampicillin.

Table 5.2 Hydrogenase activity in cell free extracts of *E. coli* strains and *C. acetobutylicum* P262.

Strain	Hydrogenase Activity	
	($\mu\text{l H}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$)	($\mu\text{mol H}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$)
<i>C. acetobutylicum</i> P262	28.18	1.152
<i>E. coli</i> Puig426 [pBR322]	4.04	0.165
<i>E. coli</i> SE53 [pBR322]	0.22	0.008
<i>E. coli</i> SE53 [pMET15B2]	1.98	0.081

activity or pyruvate-Fd-oxidoreductase activity was detected in any of the clones. To test for hydrogenase activity, plasmids were transformed into *E. coli* SE53, which is a *hydA* mutant (Lee et al., 1985). Cell extracts prepared from the parent of *E. coli* SE53, *E. coli* Puig426, were used as a positive control along with cell extracts prepared from *C. acetobutylicum* P262. *E. coli* SE53 containing plasmid pMET15B2 was the only isolate that consistently tested positive for hydrogenase activity. Crude extracts of *E. coli* SE53 [pMET15B2] had approximately half the activity of *E. coli* Puig426 extracts and only 7% of the activity of *C. acetobutylicum* P262 extracts (Table 5.2). All other clones showed hydrogenase levels comparable to the negative control which was *E. coli* SE53 containing plasmid pBR322.

5.3.4 PAGE studies

SDS-PAGE was carried out on crude extracts of the 26 clones in an attempt to determine if any obvious extra protein bands were present. A crude extract of *E. coli* F19 transformed with pBR322 was used as a control. The presence of one extra protein band was noted in each of the clones pMET7A, pMET7C and pMET14B (Fig. 5.1). Clones pMET7A and pMET7C are class I isolates, whereas clone pMET14B is a class III isolate. These extra protein bands had an apparent M_r of approximately 33, 105 and 44 kDa for clones pMET7A, pMET7C and pMET14B respectively.

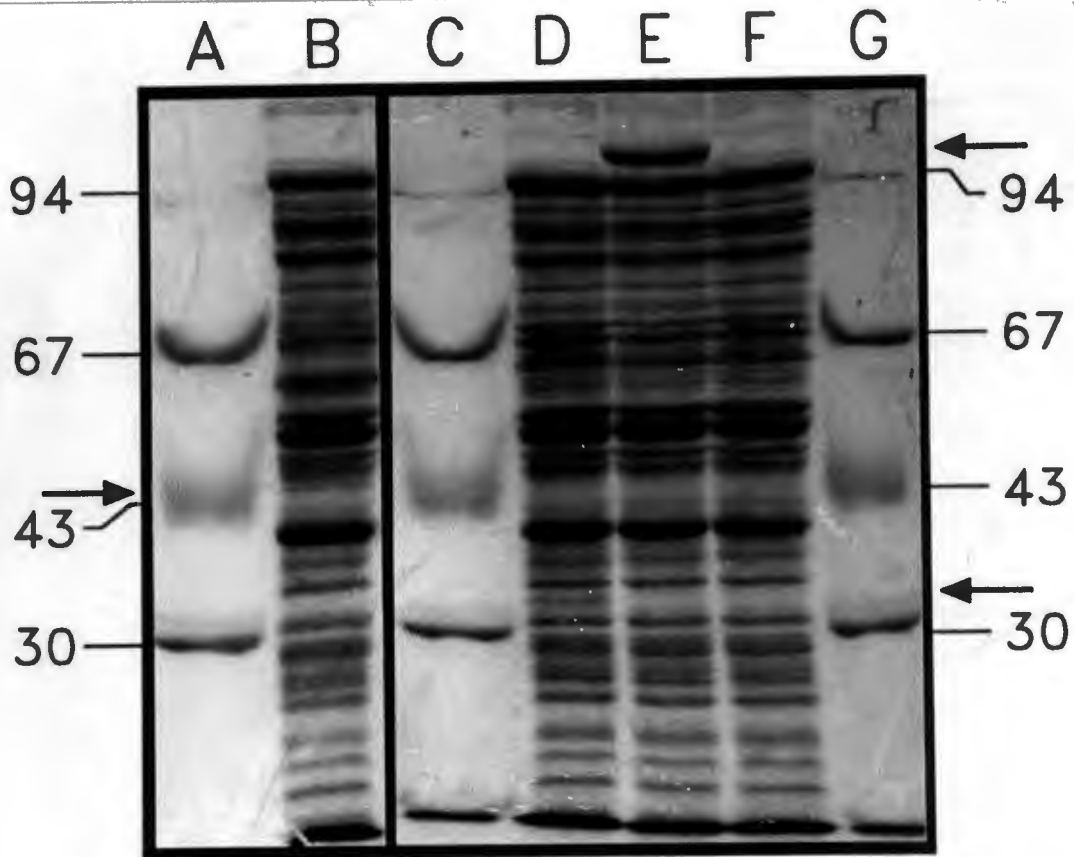


Figure 5.1 SDS-PAGE of extracts of *E. coli* F19[pMET14B] cells (lane B), *E. coli* F19[pMET7A] cells (lane D), *E. coli* F19[pMET7C] cells (lane E) and *E. coli* F19[pBR322] cells (lane F). Lanes A, C and G contained Pharmacia low molecular weight standards (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa). The arrows indicate the positions of the extra protein bands in extracts of clones pMET7A, pMET7C and pMET14B.

5.3.5 DNA hybridization studies

To determine the relatedness of the 26 clones, DNA hybridization studies were performed. Each plasmid as well as pEcoR251 with no insert, was nick translated to yield a total of 27 radiolabelled probes. Unlabelled plasmids were digested to completion with *Hin*F1 endonuclease which has a four base pair recognition sequence, and were fractionated by electrophoresis in 1.2% (w/v) agarose gels in Tris-acetate buffer. Since *Hin*F1 is a four base pair cutter, it was assumed that at least one fragment consisting of only *C. acetobutylicum* insert DNA would be generated for each plasmid. Twenty seven agarose gels were prepared, each containing *Hin*F1 digestions of all 26 plasmids plus pEcoR251. Each of these agarose gels was blotted onto a nylon membrane and probed with

Table 5.3 Results of cross hybridization studies of the twenty six plasmids isolated from the *C. acetobutylicum* gene library. All plasmids were composed of the pEcoR251 vector containing *C. acetobutylicum* insert DNA.

Plasmid Probe	Insert Size (kb)	Cross Hybridized with		
		Plasmid	Plasmid	Plasmid
pMET1A	5.15	-	-	-
pMET2D	4.90	pMET14B	-	-
pMET4A	1.20	-	-	-
pMET4B	4.30	pMET5E	-	-
pMET5E	4.30	pMET4B	-	-
pMET7A	6.35	-	-	-
pMET7C	6.05	pMET140	-	-
pMET8E	5.35	pMET13B	-	-
pMET9A	5.20	-	-	-
pMET9D	4.75	-	-	-
pMET10B	2.25	pMET040	-	-
pMET10D	6.75	-	-	-
pMET11B	7.60	-	-	-
pMET13A	6.65	pMET15B2	pMET030	pMET190
pMET13B	6.10	pMET8E	-	-
pMET13C1	4.80	-	-	-
pMET13C2	5.65	-	-	-
pMET14B	9.90	pMET2D	-	-
pMET15B2	1.00	pMET13A	pMET030	-
pMET15D	9.60	pMET130	-	-
pMET020	6.25	-	-	-
pMET030	7.05	pMET13A	pMET15B2	pMET190
pMET040	3.35	pMET10B	-	-
pMET130	4.75	pMET15D	-	-
pMET140	5.25	pMET7C	-	-
pMET190	7.95	pMET13A	pMET030	-

a different plasmid probe. Probing one of these gels with radiolabelled pEcoR251 (no insert) yielded an autoradiograph showing all DNA fragments containing vector DNA. The other plasmid probes yielded autoradiographs showing the extent of cross hybridization between the clones (Table 5.3).

From restriction endonuclease digests and these DNA hybridization studies, only isolates pMET4B and pMET5E contained identical inserts (Fig. 5.2). Therefore, of the 26 clones, 25 individual cloning events were represented. Ten of the clones hybridized only with themselves, 12 hybridized with one other clone, and 4 hybridized with two or more clones. The 12 clones that hybridized

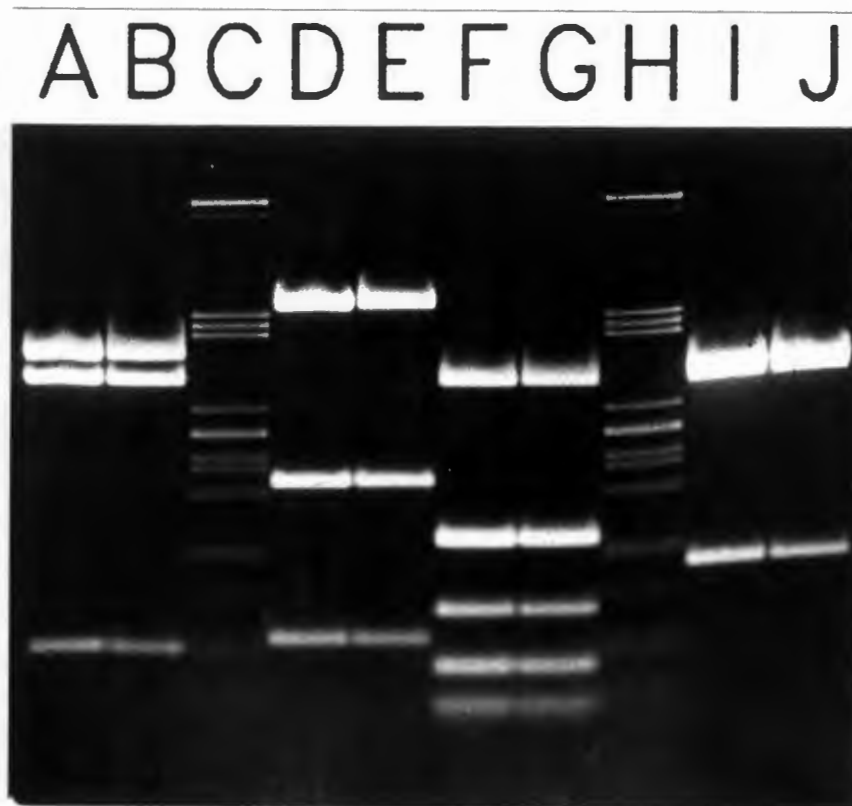


Figure 5.2 A 0.8% (w/v) agarose gel showing the identity between clones pMET4B and pMET5E. Lanes C and H contain size markers of bacteriophage λ DNA which was digested to completion with *Pst*I. Lanes A, D, F and I contain pMET4B plasmid DNA which was digested with *Hinc*II, *Pst*I, *Ssp*I and *Hin*DIII respectively. Correspondingly, Lanes B, E, G and J contain pMET5E plasmid DNA which was digested with *Hinc*II, *Pst*I, *Ssp*I and *Hin*DIII respectively. In all cases, the digestion pattern of both pMET4B and pMET5E were identical.

with one other clone may represent different size fragments of the same region of *C. acetobutylicum* DNA. Therefore, the area of *C. acetobutylicum* DNA containing the active region may be completely contained on each of the two clones or alternatively, a truncated version may exist on one of the clones. The latter may explain why there are differences in the sensitivity class of two clones that hybridized with each other. The four clones, pMET13A, pMET15B2, pMET030 and pMET190, make up the most intriguing group, ie. those clones that hybridized with 2 or more other clones. It is interesting to note that with the exception of clone pMET15B2, all of the isolates in this group have a class V sensitivity to metronidazole. This may indicate that clones pMET13A, pMET030 and pMET190 all contain the same active region from the *C. acetobutylicum* chromosome. This is supported by the fact that pMET13A, pMET030 and

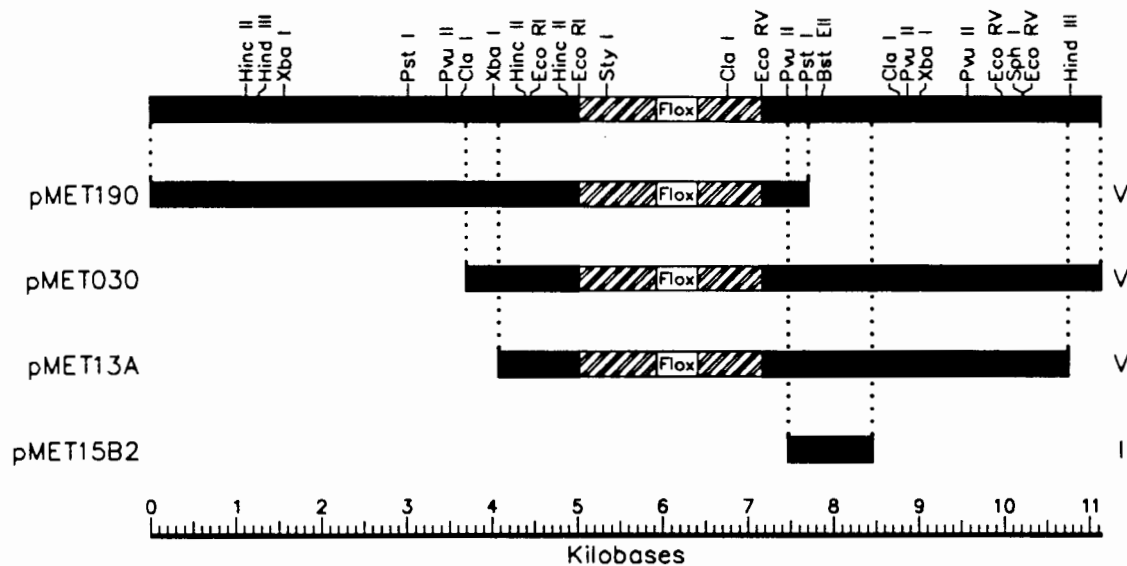


Figure 5.3 Restriction endonuclease map showing the relationship between clones pMET13A, pMET15B2, pMET030 and pMET190. The arbitrary metronidazole sensitivity class is shown on the right of each clone map. The striped region represents the area necessary to exhibit the class V metronidazole sensitivity phenotype in *E. coli* F19.

pMET190 all hybridized with each other, whereas clone pMET15B2 only hybridized with clones pMET13A and pMET030.

5.3.6 Restriction endonuclease mapping and deletion studies

The relationship between clones pMET13A, pMET15B2, pMET030 and pMET190 was determined by restriction endonuclease mapping. The restriction map of the insert DNA of these four clones is shown in Fig. 5.3. As can be seen from these restriction maps, insert DNA of clone pMET15B2 is completely contained within clones pMET13A and pMET030. Furthermore, insert DNA of clone pMET15B2 overlaps slightly with clone pMET190, however this was not identified in the hybridization studies. This is most likely due to the experimental design of the hybridizations, in that the probes used contained insert DNA as well as pEcoR251 vector DNA. The small overlap from clone pMET190 was most likely linked to pEcoR251 vector DNA and was therefore disregarded, as it hybridized with the pEcoR251 (vector only) probe. Although pMET030 and pMET13A contained the same region as pMET15B2, which showed hydrogenase activity, these two plasmids did not express hydrogenase activity in *E. coli* SE53.

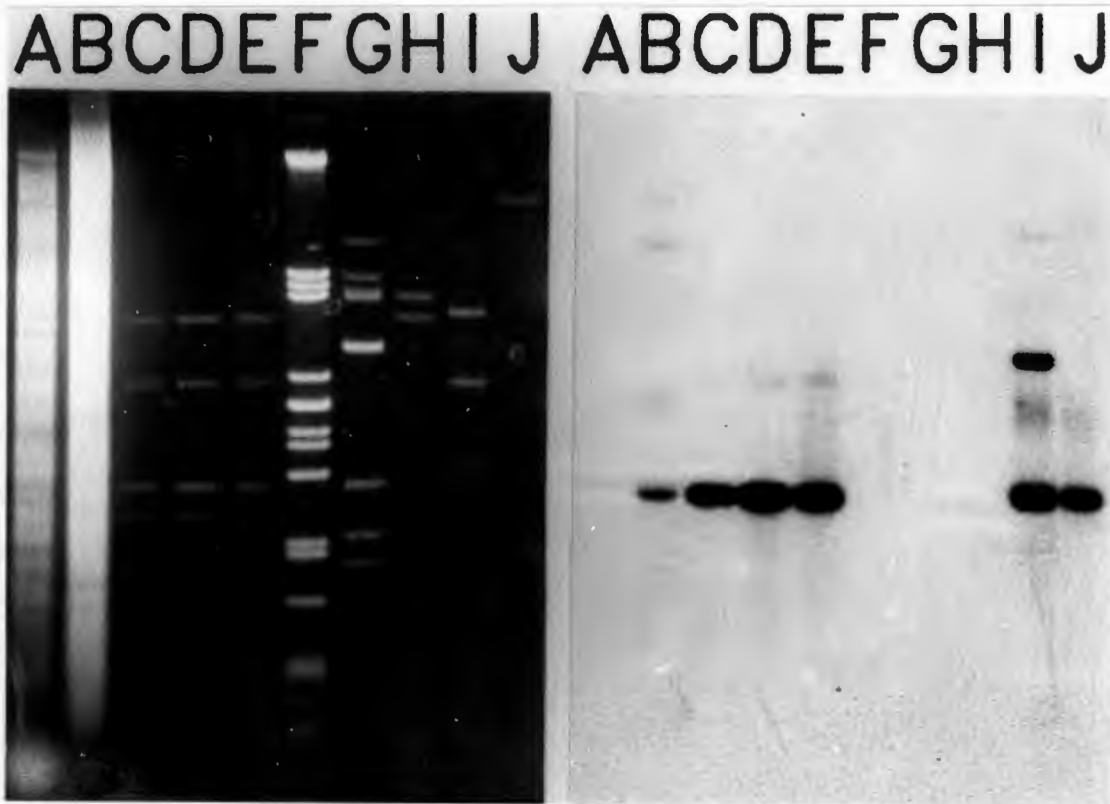


Figure 5.4 Southern blot analysis of five clones that show class V sensitivity towards metronidazole. The agarose gel is shown on the left and the corresponding autoradiograph on the right. The 1.6 kb *StyI-EcoRV* fragment from pMET13A was nick translated and used to probe against *StyI-EcoRV* digested DNA from: *E. coli* chromosome (lane A); *C. acetobutylicum* chromosome (lane B); pMET13A plasmid (lanes C, D, and E); pMET11B plasmid (lane G); pMET13C1 plasmid (lane H); pMET030 plasmid (lane I); and pMET190 plasmid (lane J). Lane F contains *PstI* digested λ DNA as size standards.

By performing restriction endonuclease deletions of the insert DNA of clone pMET13A, the region responsible for class V sensitivity to metronidazole was localized to a 2 kb *EcoRI-EcoRV* fragment (Fig. 5.3). To verify that this DNA originated from *C. acetobutylicum* P262, Southern blot analysis was performed using the *StyI-EcoRV* fragment from clone pMET13A as a probe (Fig. 5.4). Chromosomal DNA isolated from *C. acetobutylicum* and *E. coli* (negative control), as well as plasmid DNA isolated from clones pMET11B, pMET13A, pMET13C1, pMET030 and pMET190 was digested with *StyI* and *EcoRV* and the resulting fragments were separated by electrophoresis in 0.8% (w/v) agarose in Tris-acetate buffer. The *StyI-EcoRV* probe hybridized to *StyI-EcoRV* fragments of identical size from clones pMET13A, pMET030 and pMET190, as well as a

StyI-EcoRV fragment of identical size from *C. acetobutylicum* chromosomal DNA. No hybridization to the *E. coli* chromosomal DNA was demonstrated. This indicates that the region of DNA responsible for class V sensitivity in clone pMET13A is contained within clones pMET030 and pMET190, and also verifies that this DNA originated from the *C. acetobutylicum* chromosome. Plasmids pMET11B and pMET13C1 were included in this Southern blot analysis because they also demonstrated class V sensitivity to metronidazole. Since the active region from pMET13A did not hybridize with either pMET11B or pMET13C1, the class V sensitivity to metronidazole associated with these clones is separate and unique from that found on clones pMET13A, pMET030 and pMET190.

5.4 Discussion

The *E. coli* F19 *recA*, nitroreductase deficient mutant proved to be a most suitable host for the isolation of recombinant plasmids. Screening experiments resulted in the isolation of 25 different recombinant plasmids which contain *C. acetobutylicum* DNA coding for genes involved in the activation of metronidazole. The *E. coli* F19 mutant can be utilized for the isolation of genes from other bacteria which are involved in the reduction of metronidazole. Furthermore, a *recA*⁺ revertant of *E. coli* F19 has been isolated (Fig. 5.5). Therefore, a battery of *E. coli* strains and *C. acetobutylicum* genes exists for testing the activities of novel antimicrobial compounds related to metronidazole. It can be inferred whether a compound needs to be reduced to become active and whether the active compound is related to DNA repair by comparing the sensitivity of the various strains, with and without the cloned genes, to these novel compounds.

In choosing enzyme systems which may be controlled by the 25 plasmids, it was obvious that nitroreductase activity should be analyzed. However, in strict anaerobes such as *C. acetobutylicum*, where oxygen can not be utilized as the terminal electron acceptor, other electron transport mechanisms also exist. These electron transport systems typically operate at a much lower redox potential than nitroreductase systems. Ferredoxin is a low molecular weight, iron-sulfur

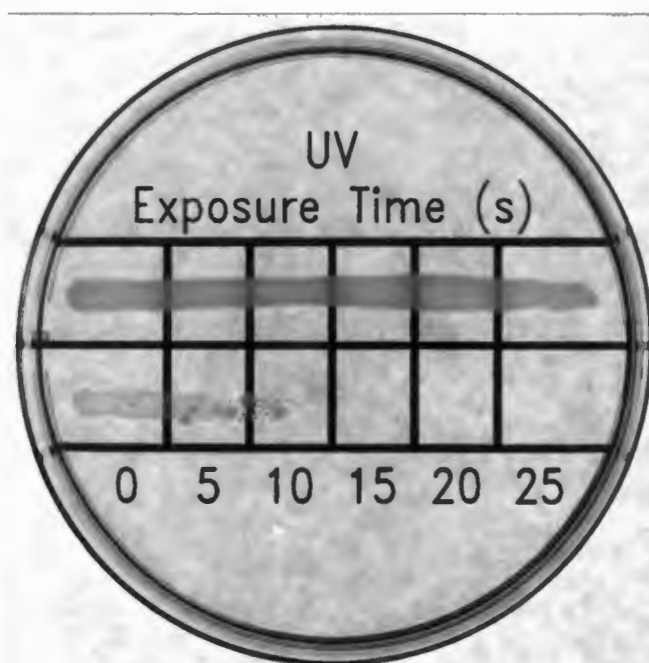


Figure 5.5 Results of a UV dose experiment demonstrating the *recA*⁻ and *recA*⁺ isolates of *E. coli* strain F19. The *recA*⁺ isolate (top streak) survived a 25 sec exposure to UV light ($1 \text{ J m}^{-2} \text{ sec}^{-1}$), whereas the *recA*⁻ isolate (bottom streak) was killed with a 10 sec exposure.

containing protein which can accept and donate electrons at a very low potential approaching that of the hydrogen electrode ($E_0' = -410 \text{ mV}$) (Jones and Woods, 1986a). This molecule plays a crucial role as an electron carrier in electron distribution in the cell. Oxidized ferredoxin accepts electrons during the phosphoroclastic conversion of pyruvate to acetyl CoA. The enzyme responsible for this conversion and electron transfer is the pyruvate-Fd-oxidoreductase. Reduced ferredoxin can transfer electrons to the iron-containing hydrogenase which uses protons as the final electron acceptor. A simplified schematic of this electron transport system is shown in Fig. 5.6. Like ferredoxin, flavodoxin is a small acidic molecule that functions as a low potential electron transfer protein (Krey et al., 1988). The redox potential of the semiquinone/hydroquinone couple is approximately -450 mV (Mayhew and Ludwig, 1975). Flavodoxin was first isolated from *C. pasteurianum* grown under iron deficient conditions (Knight et al., 1966) and it was shown to be able to replace ferredoxin as an electron carrier (Knight and Hardy, 1966; Knight and Hardy, 1967).

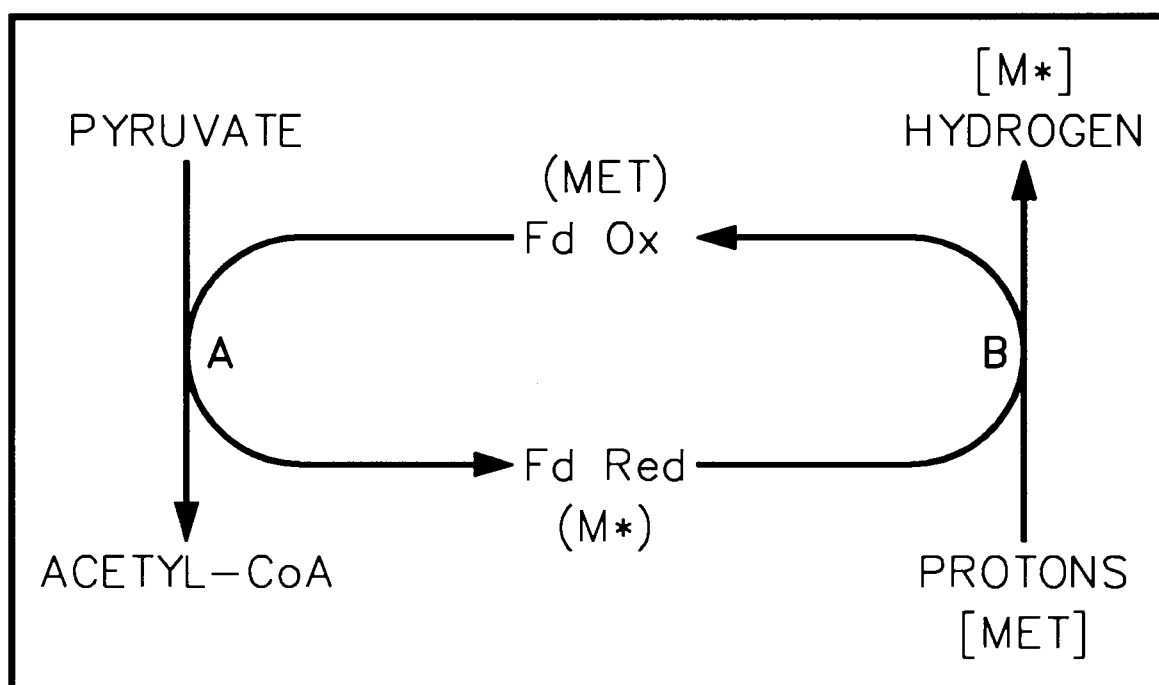


Figure 5.6 A simplified schematic of the *C. acetobutylicum* electron transport system. The relationship between oxidized ferredoxin (Fd_{ox}), reduced ferredoxin (Fd_{red}), pyruvate-Fd-oxidoreductase (A) and hydrogenase (B) is shown. In the absence of metronidazole, electrons are transferred to Fd_{ox} by pyruvate-Fd-oxidoreductase. Hydrogenase transfers electrons from Fd_{red} to protons to form hydrogen gas. In the presence of metronidazole, electrons may be transferred to metronidazole (MET) by pyruvate-Fd-oxidoreductase to form the reduced toxic derivative of metronidazole (M^*), which causes DNA damage (Narikawa, 1986). Alternatively, [MET] may replace protons and be reduced to $[M^*]$ by the action of the hydrogenase enzyme (Church et al., 1988, 1990).

The theory proposed by Narikawa (1986) that the enzyme pyruvate-Fd-oxidoreductase is responsible for the reduction of metronidazole to its active intermediates, assumes that metronidazole replaces oxidized ferredoxin in the phosphoroclastic breakdown of pyruvate to acetyl CoA (Fig. 5.6). It is therefore surprising that none of the clones isolated in this study demonstrated nitroreductase or pyruvate-Fd-oxidoreductase activity, but this does not rule out the possibility that *C. acetobutylicum* nitroreductase or pyruvate-Fd-oxidoreductase can reduce metronidazole to its active form. The assay system used to measure pyruvate-Fd-oxidoreductase activity uses the artificial electron acceptor, methyl viologen, as the indicator of enzyme activity. In principal, pyruvate-Fd-oxidoreductase transfers electrons from the breakdown of pyruvate to oxidized methyl viologen, which is colourless. This in turn forms reduced

methyl viologen which is blue in colour and can be measured spectrophotometrically at 600 nm. Unfortunately, extracts prepared from anaerobically respiring *E. coli* cells are able to reduce methyl viologen in the absence of additional pyruvate. No increase in the rate of reduction of methyl viologen was observed in extracts prepared from the isolated clones. One or more of the clones may indeed contain the gene for the pyruvate-Fd-oxidoreductase, however these can only be identified by employing more specific and sensitive techniques.

O'Brien and Morris (1972) studied the effects of metronidazole on hydrogen production in *C. acetobutylicum*. These authors reported that when sub-inhibitory concentrations of metronidazole were added to *C. acetobutylicum* cultures, hydrogen evolution ceased until the presence of metronidazole could no longer be detected. Despite the inhibitory effect of metronidazole on hydrogen production, no alteration in carbon dioxide production occurred. This indicates that electrons normally utilized for hydrogen production were siphoned away for metronidazole reduction. The lack of hydrogen production could have been caused by electrons being transferred to metronidazole instead of ferredoxin. In this case, no electrons would be available for the hydrogenase enzyme component of the *C. acetobutylicum* electron transport cycle. Alternatively, in support of the theory of Church et al. (1988, 1990), the hydrogenase enzyme could have been responsible for the reduction of metronidazole, where metronidazole replaced protons as the final electron acceptor. Another possibility is that reduced ferredoxin directly transferred electrons to metronidazole without enzyme action. In all of these cases, electrons would not be available for the production of hydrogen gas (Fig. 5.6).

The enzymes NADH- and NADPH-Fd-oxidoreductase (Jungermann et al., 1973; Jungermann et al., 1976; Petitdemange et al., 1976) may operate at a low enough redox potential to activate metronidazole. Attempts were made to assay these enzymes in *E. coli* using NAD(P)H as the indicator. Unfortunately, extracts prepared from anaerobically respiring *E. coli* cells were able to efficiently reduce

these indicator compounds, making it extremely difficult to specifically assay for these oxidoreductases.

E. coli [pMET15B2] was the only transformant that demonstrated hydrogenase activity, which was approximately half of the activity of the wild type *E. coli* strain Puig426 (Table 5.2). Voordouw et al. (1985) reported very low levels of hydrogenase activity for the *Desulfovibrio vulgaris* hydrogenase cloned into *E. coli*. *E. coli* therefore may not be a suitable host for expressing hydrogenase activity and perhaps lacks the necessary electron carriers required by the *C. acetobutylicum* hydrogenase. The fact that *E. coli* [pMET15B2] was a class I isolate may be related to the low levels of hydrogenase activity. Since only one of the 25 different recombinant plasmids encoded an enzyme that was likely to be involved in metronidazole reduction suggests that either these enzyme activities are unstable and difficult to assay in *E. coli* or there are some interesting genes encoding proteins not before considered to be involved in metronidazole reduction.

Since the insert DNA of plasmid pMET15B2 is completely contained in the insert DNA of plasmids pMET13A and pMET030, it is surprising that hydrogenase activity was not detected in cell free extracts prepared from *E. coli* SE53 transformed with these plasmids. It can be speculated that the *hydA* mutation in *E. coli* SE53 can only be complemented by pMET15B2 because it lacks the additional DNA found in pMET13A and pMET030. Another possibility is that other coding regions contained on pMET13A and pMET030 interfere with the hydrogenase assay.

DNA hybridization and restriction endonuclease mapping revealed that pMET13A, pMET15B2, pMET030 and pMET190 were linked. The total length of chromosomal DNA from *C. acetobutylicum* represented by pMET13A, pMET15B2, pMET030 and pMET190 is approximately 11.1 kb. This 11.1 kb DNA fragment from *C. acetobutylicum* contained at least two genes involved with electron transfer systems capable of activating metronidazole. The insert DNA in pMET190 and pMET13A are in opposite orientations with respect to the vector

DNA indicating that in *E. coli*, the gene responsible for metronidazole activation is most likely transcribed from a promoter present on the insert.

Restriction endonuclease deletion studies, and Southern blot analysis (Fig 5.4) revealed that the region responsible for class V sensitivity to metronidazole on clones pMET13A, pMET030 and pMET190 was contained on a 2 kb *EcoRI-EcoRV* fragment (Fig. 5.3). Since difficulties were encountered in measuring *C. acetobutylicum* electron transport enzymes in *E. coli*, and considering that this class V active region was independently cloned three times, it was decided to proceed with nucleotide sequence analysis in an effort to determine what gene product or products are coded for by this 2 kb fragment. Nucleotide sequencing and analysis of this region are the topics of the next chapter.

Chapter 6

Nucleotide sequencing and sequence analysis of selected metronidazole sensitive clones

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Chapter 6

Nucleotide sequencing and sequence analysis of selected metronidazole sensitive clones

6.0 Summary

The 2 kb *EcoRI-EcoRV* from pMET13A was shown to be the region responsible for class V sensitivity to metronidazole (Chapter 5). The nucleotide sequence of this fragment was determined and found to be 1 902 bp in length. Analyses of the sequence data revealed the presence of two truncated open reading frames (ORF1 and ORF3) and one complete open reading frame (ORF2) which was responsible for class V sensitivity of *E. coli* F19 [pMET13A]. The deduced amino acid sequence (160 amino acids) of ORF2 showed good similarity with amino acid sequences of flavodoxin proteins from many organisms. Based on alignments to the amino acid sequences of these flavodoxins, as well as the fact that Chen and Blanchard (1979) reported that reduced flavodoxin can transfer electrons to metronidazole, the sequence corresponding to ORF2 was identified as coding for a flavodoxin gene from *C. acetobutylicum*. Analyses of the deduced amino acid sequences of ORF1 and ORF3 did not result in the identification of these gene products.

Analysis of the nucleotide sequence of other cloned *C. acetobutylicum* genes resulted in the compilation of a codon usage table. Comparison of these codon usage statistics with the codon usage in the nucleotide sequence of the *EcoRI-EcoRV* fragment of pMET13A revealed that ORF1, ORF2 and ORF3 contained relatively few rare *C. acetobutylicum* codons. The presence of putative consensus promoter and termination sequences relating to these open reading frames is discussed.

Partial nucleotide sequence data of the *C. acetobutylicum* insert DNA on plasmid pMET11B is presented. *E. coli* F19 [pMET11B] also showed class V sensitivity towards metronidazole. One large open reading frame was located in this

sequence and the deduced amino acid sequence showed good similarity with *spoIID* proteins from *B. subtilis* and *B. amyloliquefaciens*

The role of flavodoxin in *C. acetobutylicum* and other organisms is presented. Possible relationships between the cloned *C. acetobutylicum* flavodoxin gene and metronidazole sensitivity are discussed.

6.1 Introduction

Chapter 5 described the isolation of 25 *E. coli* F19 clones which carried different recombinant plasmids containing *C. acetobutylicum* DNA coding for genes involved in the activation of metronidazole. These 25 clones were classified into 5 groups (I-V) based on their sensitivity towards metronidazole. The class V group, which was most sensitive to metronidazole, consisted of 5 clones of which 3 (pMET13A, pMET030 and pMET190) contained overlapping insert DNA. All three of these clones contained the 2 kb *EcoRI-EcoRV* fragment identified as being responsible for the class V activity. Since no enzyme activity could be detected in any of the class V isolates, and since the active *EcoRI-EcoRV* fragment was isolated as 3 independent cloning events, obtaining the DNA sequence of this region was deemed necessary for identification of the gene or genes responsible for metronidazole activation.

This chapter describes the DNA sequencing, characterization and amino acid homology of the 2 kb *EcoRI-EcoRV* fragment responsible for class V activity in the above 3 clones. Preliminary sequence data and analysis of another class V isolate (pMET11B) is also presented.

6.2 Materials and Methods

Commonly used materials and techniques are presented in the Appendices. The details provided below are included for completeness.

6.2.1 Chemicals

Restriction endonucleases were obtained from several sources and were used as specified by the manufacturers. The enzymes exonuclease III, S1 nuclease and DNA polymerase I Klenow fragment (PolIK) were obtained from Boehringer Mannheim Biochemicals. [α - 35 S] dATP was obtained from Amersham International. All other chemicals were analytical grade and were purchased from various local suppliers

6.2.2 Bacterial strains and plasmids

The *E. coli* strains used throughout this study are described in Appendix C. *E. coli* strain LK111 (Zabeau and Stanley, 1982) was used as the host for the exonuclease III shortened templates, whereas *E. coli* F19 was used as the host for the determination of MIC values. Plasmid pMET13A was used as the primary source of DNA. The M13 derived "BlueScript" phasmid vector (Stratagene, San Diego, CA) was used for the preparation of templates for DNA sequencing as described below.

6.2.3 Media and buffers

All media and buffers not described in the text are presented in Appendix A. The *E. coli* strains were grown as described in section 4.2.2.

6.2.4 Preparation of DNA

Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981). The small-scale (miniprep) and large-scale (maxiprep) methods of plasmid isolation are described in Appendix B. To ensure high quality preparations, plasmid DNA used for nucleotide sequencing was always purified on a cesium chloride gradient (Appendix B).

6.2.5 Sequencing strategy

Restriction endonuclease mapping and deletion studies of plasmid pMET13A (chapter 5), showed that the region responsible for class V sensitivity to metronidazole was contained on a 2 kb *EcoRI-EcoRV* restriction fragment. This *EcoRI-EcoRV* fragment was subcloned into the BlueScript vector (orientation KS) which had been linearized with *EcoRI* and *HinCII* endonucleases, to yield plasmid pSEQ13A (Fig. 6.1). The BlueScript vectors have a large multiple cloning region which contains 26 unique restriction sites. This large multiple cloning region made it possible to chose a unique restriction site for DNA shortening, using the exonuclease III technique (Henikoff, 1984, 1987). Since the active region of pMET13A had been subcloned into the middle of this large multiple cloning region, pSEQ13A was suitable for the generation of shortened fragments from both directions of the insert.

6.2.6 Exonuclease III shortening

Progressive deletions of plasmid pSEQ13A from both the 5' and 3' ends of the insert were generated by unidirectionally digesting *Bst*XI-*Bam*HI and *Kpn*I-*Cl*aI restriction endonuclease fragments with exonuclease III (Henikoff, 1984, 1987). After digestion, the linearized plasmid (approximately 12 µg) was precipitated with isopropanol, resuspended in 100 µl Exo-buffer, and equilibrated at 37°C for 5 min. Eleven microfuge tubes containing 25 µl of ice cold S1 nuclease mixture were prepared just prior to initiation of the shortening reaction and were kept on ice. A time zero sample (9 µl) was removed to a tube containing S1 nuclease mixture before exonuclease III addition. The shortening reaction was carried out at 37°C and was initiated by the addition of 300 units of exonuclease III. Samples (9 µl) were removed at 20 second intervals and immediately added to the 10 remaining microfuge tubes containing the S1 nuclease mixture. After the last sample was taken, the microfuge tubes were incubated at room temperature for 30 min for the S1 nuclease to digest single stranded DNA. The S1 nuclease reaction was stopped by the addition of 3.4 µl (per tube) of S1 stop solution, followed by incubation at 70°C for 10 min. The extent of shortening was checked by electrophoresis of approximately 200 ng of DNA (7 µl) from every second tube in 0.8% (w/v) agarose gels in Tris-acetate buffer. To facilitate the blunting of the exonuclease III generated ends, 0.5 units of PolIK was added to each tube followed by incubation for 5 min at room temperature. A further 5 min incubation was carried out in the presence of a mixture of dNTP's (0.125 mM each, A, C, G and T). The shortened DNA was recircularized by the addition of 120 µl of ligation mixture to each tube, and these tubes were incubated at 15°C for 4 h. Competent *E. coli* LK111 cells were transformed with the ligation mixtures and transformants were selected on LB agar plates containing ampicillin (100 µg ml⁻¹).

6.2.7 Nucleotide sequencing

The nucleotide sequence of both strands of the pMET13A active region was determined using overlapping DNA fragments generated by exonuclease III digestion as described above. Small scale (miniprep) preparations of

recombinant plasmids resulting from exonuclease III digestion of linearized pSEQ13A DNA were analyzed by restriction endonuclease mapping before large scale CsCl purified plasmid DNA was prepared for nucleotide sequencing. DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method developed by Sanger et al. (1977), according to the protocol of Tabor and Richardson (1987), using the Sequenase DNA sequencing kit (U.S. Biochemical Corporation, Cleveland, OH). The DNA chains were radiolabelled with [α -³⁵S]dATP (1 200 Ci mmol⁻¹). The complete sequencing protocol, including preparation of template DNA, primer annealing, sequencing reactions, gel electrophoresis and autoradiography, is described in Appendix B.

6.2.8 Sequence analysis

The nucleotide and deduced amino acid sequences were analyzed on a VAX 6000-330 computer using the Genetics Computer Group Inc. suite of sequence analysis programs (Devereux et al., 1984). The databases GenBank (release 65.0, September 1990; Bilofsky et al., 1986), EMBL (release 24.0, August 1990; Hamm and Cameron, 1986), SWISS-Protein (release 15.0, August 1990; Hamm and Cameron, 1986), NBRF-Nucleic (release 36.0, April 1990; George et al., 1986) and NBRF-Protein (release 25.0, June 1990; George et al., 1986) were searched for related nucleotide and amino acid sequences. Since the EMBL, GenBank and Swiss-protein databases are updated daily, further homology studies were later carried out via electronic mail to the GenBank file server (SEARCH@GENBANK.BIO.NET) (Yudin, 1990) and the EMBL file server (FASTA@EMBL.BITNET) (Stoehr and Omond, 1989).

6.3 Results

6.3.1 Isolation of sequencing templates

The exonuclease III shortening method was utilized to obtain overlapping shortened fragments for the sequencing of pSEQ13A insert DNA in both directions. It was necessary to subclone one small fragment (H2) to complete the sequence. Eleven overlapping clones were required to complete the sequence of the coding strand, and similarly, 11 clones were necessary to complete the

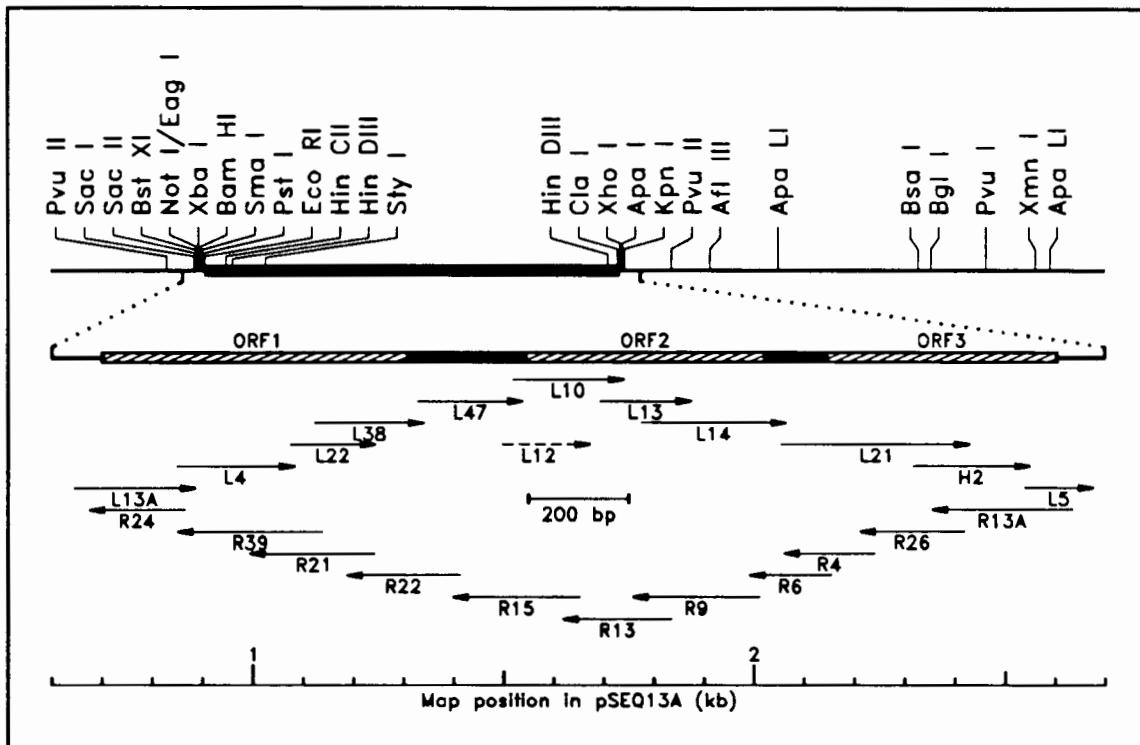


Figure 6.1 Sequencing strategy for plasmid pSEQ13A containing insert DNA from *C. acetobutylicum* P262. The insert DNA is indicated by the bold line and the thin line represents BlueScript KS vector DNA. The arrows indicate the extent of sequence obtained from each clone. Clone names are written below each arrow. Clone H2 was constructed from a *Hin*FI-*Xho*I fragment of clone L21. This fragment was subcloned into BlueScript KS vector that had been restricted with *Eco*RV and *Xho*I. This subclone was necessary to complete the sequence of the coding strand. The three identified open reading frames ORF1, ORF2 and ORF3 are indicated by the hatched regions. The clone L12 (indicated by a dashed line) was not necessary to complete the sequence of the coding strand, but was used to locate the region responsible for metronidazole activation.

sequence of the non-coding strand. The extent of the sequence obtained from each clone is depicted in Fig. 6.1.

6.3.2 DNA sequence data

The nucleotide sequence of the *Eco*RI-*Eco*RV active region of pMET13A was determined to be 1 902 bp in length, and is shown in Fig 6.2. The nucleotide sequence of this fragment showed that two partial open reading frames (ORF1 and ORF3 in Figs. 6.1 and 6.2) and one complete open reading frame (ORF2 in Figs. 6.1 and 6.2) were present. The first partial open reading frame (encoding 159 amino acids) was truncated by the *Eco*RI site, and similarly the second partial


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      720      740      760      780
EcoRI
GA ATT CCT TTT ATT ATT CCA GTC AAT ACA GAG CCA GAA ATA ATT AAA GCT CAA TTA GAA AAT GTA GAT GGA CTT ATT TTA TCA GGT GGT
I P F I I P V N T E P E I I K A Q L E N V D G L I L S G G

      800      820      840      860      880
CAT GAT GTT AAC CCA TTA TTA TTT AAT GAA GAA CCA CAT AAG CTT TTG GGA AAT ACA ATG AAT GAA AGA GAC ACA TTT GAT TCT ATG TTA
H D V N P L L F N E E P H K L L G N T M N E R D T F D S M L

      900      920      940      960
ATA AAA TAT GCA TTT GAA ATG GAA AAG CCT ATC TTA GGT ATT TGT AGG GGA TGC CAA ATT TTA AAT GTT GTA TGT GGT GGA ACA TTA TAT
I K Y A F E M E K P I L G I C R G C Q I L N V V C G G T L Y

      980      1000      1020      1040      1060
CAA GAT TGT TCC TTG GCA AAG GAT TCG TAT ATA AAA CAT TGT CAA GGG CAT ACA CCA GCA CAA CCT TCT CAT ACG ATA CAG ATT GAA AAA
Q D C S L A K D S Y I K H C Q G H T P A Q P S H T I Q I E K

      1080      1100      1120      1140
AAT TCT GAA TTA TAT ACT ATT TTA GGA GAA AAA GCT CTT GCA AAT TCA TTT CAT CAT ATG TCT ATA AAA GAT GTT GCG GAT GGA TTT AAG
N S E L Y T I L G E K A L A N S F H H M S I K D V A D G F K

      1160      1180      1200      1220      1240
GTA GTA GCT ATA GCT AAT GAT GAA ATT ATT GAA GCA ATA GAG AAG GTA GAT GGT TCT TTT GCA CTT GGA ACA CAA TGG CAT CCA GAA ATG
V V A I A N D E I I E A I E K V D G S F A L G T Q W H P E M

      1260      1280      1300      1320
ATG GCA AGC AGT GAT GAA AAT ATG CTG AAA ATA TTT AAA TTA TTT ATA TCT AAA TGT AAG AAT AGT AGA TTA TAG AATGAGTAAAAATAATTT
M A S S D E N M L K I F K L F I S K C K N S R L *

      1340      1360      1380      1400      1420      1440
GAATTTTAAATTATATAGTTACTGAGTATTAATATTCATGATAAAAAATGATAGCAAAACTAAATGTTACTATAAATTATTTTGGTATTAACAAAAGCATTGTGCATGATGTGGA
1460      1480      1500      1520      1540      1560
ATTATTAATTCAGATGATACAATAAGTCTAAAAATATACAATTTAATTATTTTAAACAATATAGTTTAAATTAAGGATTTTATCA ATG AAA ATA TCA ATA CTA TAC
M K I S I L Y

      1580      1600      1620      1640
AGT AGC AAG ACA GGA AAA ACC GAA AGA GTT GCC AAG TTA ATT GAA GAA GGT GTG AAG AGG TCT GGA AAT ATT GAA GTA AAA ACT ATG AAT
S S K T G K T E R V A K L I E E G V K R S G N I E V K T M N

      1660      1680      1700      1720      1740
TTA GAT GCA GTT GAT AAA AAG TTC TTA CAA GAA TCA GAA GGA ATT ATT TTT GGA ACA CCA ACT TAT TAT GCC AAC ATA TCA TGG GAA ATG
L D A V D K K F L Q E S E G I I F G T P T Y Y A N I S W E M

      1760      1780      1800      1820
AAA AAA TGG ATA GAT GAA TCA TCA GAA TTT AAT TTA GAA GGA AAA CTT GGA GCA GCA TTT TCA ACA GCT AAC TCT ATT GCT GGT GGT TCA
K K W I D E S S E F N L E G K L G A A F S T A N S I A G G S

      1840      1860      1880      1900      1920
GAT ATA GCA TTA CTT TCA ATT TTA AAT CAT CTT ATG GTA AAA GGA ATG TTA GTA TAT TCA GGT GGA GTT GCT TTT GGA AAA CCA AAG ACT
D I A L L T I L N H L M V K G M L V Y S G G V A F G K P K T

      1940      1960      1980      2000
CAT TTA GGA TAT GTG CAT ATA AAT GAA ATA CAA GAA AAT GAA GAT GAA AAT GCG AGA ATA TTT GGT GAA AGA ATT GCA AAC AAA GTC AAA
H L G Y V H I N E I Q E N E D E N A R I F G E R I A N K V K

      2020      2040      2060      2080      2100      2120
CAA ATA TTT TAA TGTAAAAATGTATATATAAATTGATTAATAATTTAAAAATATACAATATTATAATATTGAGAGCGAAGTTTATAATATGTTATAAATTAATAAATAGATGG
Q I F *

      2140      2160      2180      2200      2220
AAGGAAATGTTATT ATG AAT GAA GTA TTA CAA AAT ATT TTA ACA AGA AGA AGT GTA AGA AAA TTT AAA CAA GAA CAA ATA AAA GAT GAG GAA
M N E V L Q N I L T R R S V R K F AAA CAA GAA CAA Q I K D E E

      2240      2260      2280      2300
CTA GAT TTA ATA TTA AAA GCT GGA GTA TAT GCA CCA AGT GGT ATG AAC AAG CAA AGT TGG CAG TTT ACA GTT GTT CAA AAT AAA GAA AAA
L D L I L K A G V Y A P S G M N K Q S W Q F T V V Q N K E K

      2320      2340      2360      2380      2400
ATA GAA TCT CTT GCT AAA GTA GTC AGG GAA GCA TTA GGT AGA GAT GCA GGA TAT AAT TTT TAT GCA CCT CCT ACT TTA ATT ATG TTA TCT
I E S L A K V V R E A L G R D A G Y N F Y A P P T L I M L S

      2420      2440      2460      2480
AAT GAC AAA GAA AAT ACT AAT GGA TTG GCA GAT TGT TCT TGT GCA TTA GAA AAT ATA TTC TTA ATG GCT AAT TCA TTG GGT ATA GGT TCA
N D K E N T N G L A D C S C A L E N I F L M A N S L G I G S

      2500      2520      2540      2560      2580
TGT TGG ATT AAT CAA TTG AAA ACT ATT TGT GAT GAA AAG GAA GTA AGA GAA TTA TTA ACA AGC TTT GGA ATA CCA GAA AAC CAT ATT GTA
C W I N Q L K T I C D E K E V R E L L T S F G I P E N H I V

      2600
TGG GGA ATG GCA TCG ATT GGA TATc
W G M A S I G
EcoRV

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open reading frame (164 amino acids) was truncated by the *EcoRV* site. Further nucleotide sequence is necessary to complete these open reading frames. The complete open reading frame (160 amino acids) that lies between the two truncated open reading frames, is preceded by a 230 nucleotide intergenic region and followed by an 88 nucleotide intergenic region. This nucleotide sequence has been assigned the GenBank DNA sequence database accession number M36770.

To determine the minimum sequence required for the activation of metronidazole, the plasmids resulting from exonuclease III shortening of pSEQ13A were transformed into *E. coli* F19 and tested for their ability to activate metronidazole. Loss of the ability to activate metronidazole was obtained with upstream shortenings after position 1 497 (shortened clones L12 through L5), which was 46 bp upstream from the ATG start codon for ORF2 (Figs. 6.1 and 6.2). In the opposite direction, shortened clones R9 through R24 were unable to activate metronidazole. Shortened clone R9 was missing DNA from position 2 012 and resulted in the deletion of the last 4 amino acids of the protein product coded for by ORF2. This clearly indicated that ORF2 was responsible for the activation of metronidazole.

6.3.3 Codon usage

The GenBank database (release 65) contains six nucleotide sequences from *C. acetobutylicum*, and their locus names are as follows: CLOADH (Youngleson et al., 1989b), CLOBHBD (Youngleson et al., 1989a), CLOCBA (Hancock et al., 1991), CLOEGLA (Zappe et al., 1988), CLOGLNSA (Janssen et al., 1988) and CLOXYNB (Zappe et al., 1990). Furthermore, Gerischer and Dürre (1990) have submitted the nucleotide sequence for an acetoacetate decarboxylase (CLOADC) for inclusion in release 66 of the GenBank database. From the seven nucleotide sequences mentioned above, eight genes have been positively identified: an alcohol dehydrogenase (CLOADH), a β -hydroxybutyryl CoA dehydrogenase (CLOBHBD), a β -galactosidase (CLOCBA), a β -galactosidase regulatory protein (CLOCBA), an endoglucanase (CLOEGLA), a glutamine synthetase

(CLOGLNSA), a xylanase (CLOXYNB) and an acetoacetate decarboxylase (CLOADC). Using the coding regions for these eight genes, a codon frequency table was constructed (Table 6.1). The codon usage of these 8 genes reveals a bias towards the use of codons in which A and U predominate. In these 8 genes, 11 codons (GCC, CGG, CGC, CUG, CUC, CCG, CCC, UCG, UCC, ACG, GUC) were used less than 5% of the time as compared to synonymous codons coding for the same amino acid. In the 3 identified open reading frames ORF1, ORF2 and ORF3, 46, 40 and 41 of the possible 61 codons were utilized respectively. Furthermore, in all three of these open reading frames, only 35 codons were used more than once, and 9 codons (CGC, CGA, CGU, CGC, GGC, CAC, CUC, CCG, CCC) were not used at all. It is also interesting to note that in the 8 genes contained in the GenBank database, as well as in the 3 identified open reading frames from pSEQ13A, the stop codon UGA was never used to terminate the reading frame.

Table 6.1 Codon usage of 8 known *C. acetobutylicum* genes as compared to the 3 identified open reading frames of pSEQ13A.

aa	COD ^a	Identified <i>C. acetobutylicum</i> genes ^b								Totals		pSEQ13A ORFs		
		A	B	C	D	E	F	G	H	Sum	% ^c	1	2	3
Ala	CCG	4	0	3	0	0	1	1	3	12	6.0	1	1	0
	GCA	12	9	20	2	6	24	8	8	89	44.3	7	5	7
	GCU	18	21	10	0	21	15	3	6	94	46.7	4	3	3
	GCC	1	0	1	0	2	0	1	1	6	3.0	0	2	0
Arg	AGG*	0	1	2	2	0	0	0	2	7	6.6	1	1	1
	AGA*	9	9	27	1	3	19	4	4	76	71.0	2	3	5
	CGG*	0	0	0	0	0	0	0	0	0	0.0	0	0	0
	CGA*	0	0	4	2	0	0	2	1	9	8.4	0	0	0
	CGU	0	0	5	0	2	0	1	3	11	10.3	0	0	0
	CGC	0	0	1	1	0	0	1	1	4	3.7	0	0	0
Asn	AAU*	13	4	46	4	27	29	13	8	144	80.9	11	7	10
	AAC	1	4	8	3	4	6	7	1	34	19.1	1	3	2
Asp	GAU	18	15	51	3	20	26	5	17	155	85.2	10	5	5
	GAC	2	1	7	3	5	4	4	1	27	14.8	1	0	1
Cys	UGU	4	2	4	1	2	5	2	3	23	65.7	5	0	4
	UGC	1	1	3	3	2	1	1	0	12	34.3	1	0	0
Gln	CAG	0	0	10	0	6	0	6	2	24	27.6	1	0	1
	CAA	9	6	19	1	11	11	4	2	63	72.4	6	3	6
Glu	GAG	2	0	15	0	2	3	2	6	30	14.6	2	0	1
	GAA	30	22	63	3	11	31	7	8	175	85.4	14	15	12

Gly	GGG*	1	0	8	2	0	1	1	2	15	7.2	1	0	0
	GGA*	14	20	32	1	15	23	9	10	124	59.6	7	10	6
	GGU	8	4	13	0	8	5	5	1	44	21.2	5	5	4
	GGC	3	1	3	0	6	3	9	0	25	12.0	0	0	0
His	CAU	10	1	11	0	5	8	1	5	41	75.9	8	3	1
	CAC	1	1	7	0	1	0	1	2	13	24.1	0	0	0
Ile	AUA*	9	4	24	5	23	3	6	7	81	38.0	9	9	6
	AUU	16	16	30	3	10	15	7	10	107	50.2	11	7	6
	AUC	2	8	5	1	2	3	3	1	25	11.8	1	0	0
Leu	UUG*	1	1	9	1	3	1	3	3	22	9.9	2	0	3
	UUA*	23	12	28	5	12	32	6	10	128	57.4	12	8	11
	CUG	0	0	2	1	1	1	0	1	6	2.7	1	0	0
	CUA	6	1	8	1	0	4	0	0	20	9.0	0	1	1
	CUU*	2	5	16	1	4	1	4	9	42	18.8	4	3	1
	CUC*	0	0	1	1	2	0	0	1	5	2.2	0	0	0
Lys	AAG	14	7	21	5	8	7	5	6	73	30.8	6	5	2
	AAA	20	17	51	4	24	25	13	10	164	69.2	9	11	9
Met	AUG	16	11	22	4	11	13	6	9	92	100.0	7	5	5
Phe	UUU	15	7	34	1	17	16	8	7	105	80.1	9	6	4
	UUC	4	4	9	1	1	5	2	0	26	19.9	0	1	1
Pro	CCG	1	0	5	0	0	0	0	0	6	4.9	0	0	0
	CCA*	15	6	18	1	6	17	4	6	73	59.3	6	2	2
	CCU*	3	2	10	2	9	3	3	8	40	32.5	3	0	2
	CCC*	0	0	0	0	0	0	0	4	4	3.3	0	0	0
Ser	AGU*	2	0	10	3	12	3	13	2	45	22.1	2	1	3
	AGC	0	3	7	2	4	0	5	5	26	12.7	1	1	1
	UCG*	1	0	0	1	1	1	0	1	5	2.4	1	0	1
	UCA*	13	12	24	2	19	4	6	1	81	39.7	2	8	2
	UCU	2	1	12	4	14	6	1	2	42	20.6	6	2	3
	UCC	0	0	2	1	1	0	1	0	5	2.5	1	0	0
Thr	ACG	0	0	3	0	0	0	4	2	9	4.5	1	0	0
	ACA*	16	5	23	4	21	12	12	6	99	49.0	6	4	3
	ACU	8	8	17	2	26	8	10	4	83	41.1	1	3	3
	ACC	0	0	2	2	2	0	4	1	11	5.4	0	1	0
Trp	UGG	2	1	21	0	10	4	7	1	46	100.0	1	2	3
Tyr	UAU	14	6	51	6	16	13	13	13	132	83.5	4	4	3
	UAC	1	1	9	1	6	4	3	1	26	16.5	0	1	0
Val	GUG	2	0	7	1	0	1	1	2	14	7.9	0	2	0
	GUA	12	8	20	0	10	13	4	7	74	41.6	5	3	6
	GUU	6	14	23	3	12	12	6	6	82	46.1	3	3	2
	GUC	1	0	0	0	2	1	3	1	8	4.4	1	1	1
END	UGA	0	0	0	0	0	0	0	0	0	0.0	0	0	0
	UAG	0	0	1	0	0	0	0	0	1	12.5	1	0	0
	UAA	1	1	0	1	1	1	1	1	7	87.5	0	1	0

^a *E. coli* rare codons are marked by an asterisk (Ernst, 1988).

^b Identified *C. acetobutylicum* genes are as follows: A, alcohol dehydrogenase (Youngleson et al., 1989b); B, β -hydroxybutyryl CoA dehydrogenase (Youngleson et al., 1989a); C, β -galactosidase (Hancock et al., 1991); D, β -galactosidase regulatory protein (Hancock et al., 1991); E, endoglucanase (Zappe et al., 1988); F, glutamine synthetase (Janssen et al., 1988); G, xylanase (Zappe et al., 1990); H, acetoacetate decarboxylase (Gerischer and Dürre, 1990).

^c % total is expressed as the percentage of use within the group of synonymous codons.

It has been suggested that codon preference represents a bias for abundant tRNAs, thereby facilitating the biosynthesis of high levels of proteins utilizing these codons (Bennetzen and Hall, 1982; Sharp et al., 1986; de Boer and Kastelein, 1986). In *E. coli* the following codons are regarded as minor codons and are rarely utilized: GGA (Gly), UUA (Leu), AAU (Asn), CCA (Pro), AGA (Arg), UCA (Ser) and ACA (Thr) (Konigsberg and Godson, 1983; Allf-Steinberger, 1984; Ernst, 1988). In the 8 genes contained in the GenBank database, these codons were used preferentially over all other synonymous codons coding for the same amino acids. Despite the use of a high percentage of *E. coli* rare codons by these cloned genes, enough protein was produced to allow for detection via enzyme assays. Similarly ORF2 used a large percentage of *E. coli* rare codons, however this did not effect the ability of the *E. coli* clones pMET13A, pMET030 and pMET190, to produce enough protein product to allow for the metronidazole sensitive phenotype.

Using the computer program CodonPreference (Devereux et al., 1984) in conjunction with the constructed codon frequency table for *C. acetobutylicum* (Table 6.1), the graphs and diagrams shown in Fig. 6.3 were assembled. Rare codons were determined according to protocol described by Gribskov et al. (1984) and the third position compositional bias was calculated as described by Bibb et al. (1984). Fig. 6.3 shows that rare *C. acetobutylicum* codons are used less frequently in the identified open reading frames ORF1, ORF2 and ORF3, than in the intergenic regions. The compositional bias for each reading frame is the fraction of the third position in each codon that is either A or T, using a sliding window of 100 nucleotides. From these AT bias plots, it can be seen that the three identified open reading frames ORF1, ORF2 and ORF3, all have above average values for a bias towards codons that have an A or U in the third position. The opposite is seen for the non-coding regions of this sequence.

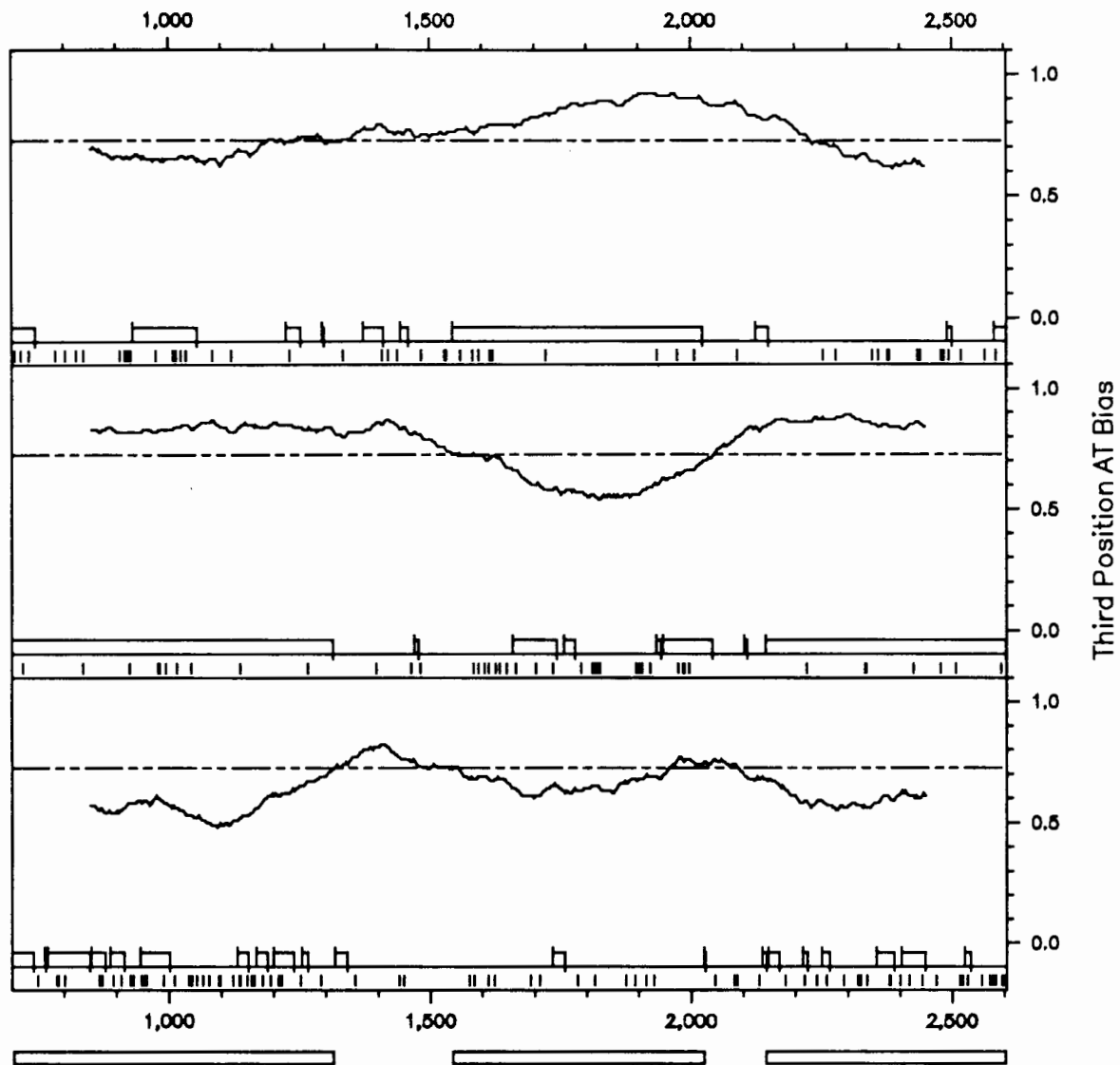


Figure 6.3 Third position AT bias and open reading frame display for the three forward reading frames of pSEQ13A. The dashed horizontal line in each of the three plots represents the mean AT bias for this sequence. All possible open reading frames are shown as boxes beneath the plot for their respective reading frames. Start codons are shown as short lines that extend above the height of the box, and stop codons are marked by lines that extend below the bottom of the box. Only the start and stop codons at the beginning and end of an open reading frame are displayed. *C. acetobutylicum* rare codons in each reading frame are marked below the open reading frame display by short vertical lines. A rare codon threshold (p) of 0.10 was used for these plots [see Gribskov et al., (1984) for an explanation of the calculation of p]. The positions of the three open reading frames ORF1, ORF2 and ORF3 are marked as boxes below the X-axis.

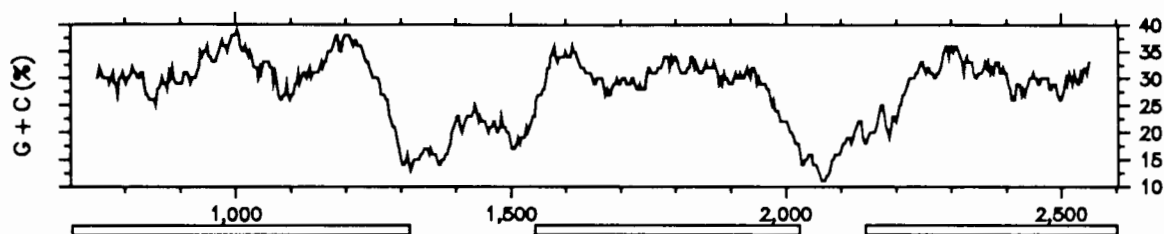


Figure 6.4 The percent G + C in a sliding window of 100 nucleotides over the length of the *C. acetobutylicum* insert DNA contained on plasmid pSEQ13A. The window was shifted along the sequence in 3 nucleotide steps. The positions of the three identified open reading frames ORF1, ORF2 and ORF3 are marked as boxes below the X-axis.

6.3.4 G + C content

The overall G + C content of the seven nucleotide sequences contained in the GenBank database is 28.70 (CLOADH), 30.76 (CLOBHBD), 30.45 (CLOCBA), 30.33 (CLOEGLA), 30.31 (CLOGLNSA), 31.57 (CLOXYNB) and 33.84% (CLOADC). These values are slightly higher than the overall G + C content for *C. acetobutylicum* which is 28% (Cummins and Johnson, 1971). The overall G + C content for the *C. acetobutylicum* insert DNA contained in plasmid pSEQ13A was determined to be 28.07%. Calculating the percent G + C in a sliding window of 100 nucleotides, which is moved along the sequence in 3 nucleotide steps, resulted in the plot shown in Fig. 6.4. The G + C content dropped dramatically in the intergenic regions between the three identified open reading frames. The G + C content of ORF1, ORF2 and ORF3 is 30.72, 30.43 and 30.06% respectively, whereas the G + C content of the intergenic regions between these open reading frames drops to 18.34 and 15.00% respectively. As mentioned in the previous section, *C. acetobutylicum* preferred codons were biased towards an A or T in the third position (Fig. 6.3), which is contrary to the overall G + C content being higher in the coding regions. The higher G + C content in the coding regions must therefore reflect the necessity for G's and C's in the first two positions of the codons in order to allow for translation to include all amino acids.

The coding regions of the seven sequences contained in the GenBank database also have higher G + C values when compared to the overall values given above. The G + C values for the coding regions of these seven sequences are as follows:

32.56 (CLOADH), 33.92 (CLOBHBD), 32.70 (CLOCBA), 32.74 (CLOEGLA), 31.76 (CLOGLNSA), 37.53 (CLOXYNB) and 37.01% (CLOADC). Since the *C. acetobutylicum* nucleotide sequences contained in the GenBank database all resulted from the isolation of known coding regions, they contain relatively small amounts of non-coding sequence. This could account for the slightly higher overall G + C values of these sequences as compared to the 28% reported by Cummins and Johnson (1971).

6.3.5 Putative RBS, promoter and termination sequences

The process of translation, in which the mRNA nucleotide sequence is translated into an amino sequence, is initiated by the interaction between the 16S ribosomal RNA (rRNA) and a specific sequence on the mRNA known as the ribosome binding site (RBS). In *E. coli* and *B. subtilis*, ribosome binding sites are generally characterized by the start codon and a purine rich (SD) sequence 4 to 15 nucleotides upstream of the start codon (Shine and Dalgarno, 1974). The consensus SD sequence 5'-AGGAGG-3' is complementary to the 3' end of 16S rRNA (Gold et al., 1981, Gold, 1988). The start codon is usually AUG, but prokaryotic ribosomes occasionally start at a GUG or UUG codon (Kozak, 1983). It has been shown that *B. subtilis* and other Gram positive organisms have a much higher frequency for non-AUG initiation codons than *E. coli* (Hager and Rabinowitz, 1985).

The ribosome binding sites of both ORF2 and ORF3 were characterized by the presence of a SD region and a AUG start codon. The AUG start codon of ORF2 is located at position 1 543 and is preceded by a putative SD region (AGGAGG) 9 bp upstream of the AUG codon (Fig. 6.2). This putative SD site resembled that reported for the *S. aureus* β -lactamase gene (McLaughlan et al., 1981) and the *C. acetobutylicum* endoglucanase gene (Zappe et al., 1988) (Fig. 6.5). The AUG start codon of ORF3 is located at position 2144 and is preceded by a putative SD site (AGGAAG) 8 bp upstream of the AUG codon (Fig. 6.2).

In Gram positive organisms such as *B. subtilis*, transcription of genes expressed during growth are dependent on the σ factor identified as σ^{43} (Graves and

<i>C. acetobutylicum</i>	ORF2	5' <u>A G G A G G</u> A T T T T A T C A 3'
<i>S. aureus</i>	<i>bla</i>	5' <u>A G G A G G</u> A G A T A A A A 3'
<i>C. acetobutylicum</i>	<i>eglA</i>	5' <u>A G G G G G</u> T A T T A A C 3'

Figure 6.5 Putative ribosome binding site of the pSEQ13A ORF2 as compared to the ribosome binding sites for the *S. aureus* β -lactamase (*bla*) gene and the *C. acetobutylicum* endoglucanase (*eglA*) gene. The Shine-Dalgarno region is underlined. In all three cases, a start codon follows immediately after the last nucleotide shown.

Rabinowitz, 1986). Promoter regions for σ^{43} dependent transcription show structural similarities with the *E. coli* σ^{70} promoters (see Fig 6.6). It is apparent that besides the traditional core regions of these Gram positive promoters, upstream and downstream regions also display conserved sequences. It is these upstream and downstream regions that have given rise to the term "extended Gram positive promoters" (Graves and Rabinowitz, 1986). Conservation of nucleotide sequences in the extended regions can compensate for a lack of conservation in the core regions. For example, a TG pair starting 3 base pairs upstream of the TATAAT Pribnow box has been shown to be sufficient to compensate for the absence of a specific -35 region (Ponnambalam et al., 1986; Keilty and Rosenberg, 1987).

As mentioned earlier (Chapter 5), the insert DNA in pMET190 and pMET13A are in opposite orientations with respect to the vector DNA indicating that in *E. coli*, the gene responsible for metronidazole activation (ORF2) is most likely transcribed from a promoter present on the insert. The high A + T content of the intergenic regions between the three open reading frames on the pSEQ13A insert DNA makes it very difficult to identify possible promoters from the sequence data alone. Speculation leads to 10 possible promoters for the 5' upstream region of ORF2 and 4 possible promoters for the 5' upstream region of ORF3 (Fig. 6.6). The location of these promoters with respect to the ribosome binding site, as well as the distance between the -10 and -35 regions, easily eliminates some of them as candidates, however they are all included as a matter of completeness. Another

EC	a	t	<u>TTGAC</u> a	t	t	t	tg	<u>TatAAT</u>	
G+	TA	AAAAA	<u>TTGAC</u> a		a	A	a	T	<u>TG</u>
Gene			-35					-10	
EG1	TTTTATATATAT	TTGTAT	TAAATTA	-----	ACTTTAATGT	TACAAT	GTTCT		
EG2	AATAAAAA	TTATTA	ATGTAAA	----	AATATACTAAGTA	TAGAAT	AATAG		
GS1	TAAAAAA TCAC	TTGATT	TCTTAAA	-----	AAAAAGGGAAG	TATAAT	TTAGT		
GS2	TGAGCAA AGGCG	TCCGAT	AGAAGTT	-----	TATACTTGTC	TATTGT	GCGCT		
Position			-35		ORF2			-10	
1485-1531	AAAATATACAAT	TTAATT	ATTTTAA	-----	ACAATATAGTT	TTTAAT	TAGGA		
1457-1501	ATTAATTCCAAG	ATGATA	CAATAAG	-----	TCTAAAATA	TACAAT	TTAAT		
1450-1499	TGGAATTATTAA	TTCCAA	GATGATA	---	CAATAAGTCTAAAA	TATACA	ATTTA		
1446-1494	GATGTGGAATTA	TTAATT	CCAAGAT	----	GATACAATAAGTC	TAAAAT	ATACA		
1438-1483	TTGTGCATGATG	TGGAAT	TATTAAT	-----	TCCAAGATGA	TACAAT	AAGTC		
1414-1466	ATTATTTTGGTA	TTAACA	AAAGCAT	-----	TGTGCATGATGTGGAAT	TATTAA	TTCCA		
1414-1460	ATTATTTTGGTA	TTAACA	AAAGCAT	-----	TGTGCATGATG	TGGAAT	TATTA		
1390-1434	GCAAAAACTAAA	ATGTTA	CTATAAA	-----	TTATTTTGG	TATTAA	CAAAA		
1372-1416	ATGATAAAAAAA	ATGATA	GCAAAAA	-----	CTAAAAATGT	TACTAT	AAATT		
1351-1398	AGTTACTGAGTA	TTAATA	TTCATGA	----	TAAAAAAAATGA	TAGCAA	AAACT		
Position			-35		ORF3			-10	
2070-2116	AATATTATAATA	TTGAGA	GCGAAGT	-----	TTATAATATGT	TATAAA	TTAAT		
2062-2106	AAATATACAATA	TTATAA	TATTGAG	-----	AGCGAAGTT	TATAAT	ATGTT		
2038-2085	TATATAAATTGA	TTAATA	ATTTAAA	-----	AATATACAATAT	TATAAT	ATTGA		
2034-2082	TGTATATATAAA	TTGATT	AATAATT	----	TAAAAATATACAA	TATTAT	AATAT		

Figure 6.6 Possible Gram positive consensus promoter sequences for ORF2 and ORF3. The top panel shows two promoter consensus sequences: EC, the *E. coli* σ^{70} consensus sequence (McClure, 1985) and G+, the extended consensus sequence reported to be a feature of the σ^{43} RNA polymerase recognition site of *B. subtilis* and other Gram positive bacteria (Graves and Rabinowitz, 1986). In this panel, highly conserved bases (>70%) are indicated by underlined capital letters; >50% conservation are capital letters and >41% conservation are lower case letters (after Graves and Rabinowitz, 1986). The second panel shows the sequence of four identified *C. acetobutylicum* promoters. The abbreviations are as follows: EG1 and EG2 are two promoters identified for the endoglucanase gene (Zappe et al., 1988), and GS1 and GS2 are two promoters identified for the glutamine synthetase gene (Janssen et al., 1990). The third and fourth panels are the positions and sequences of possible σ^{43} type promoters for ORF2 and ORF3 respectively. The sequence positions correspond to Fig. 6.2. Nucleotides with identity to the consensus sequence are shown in bold typeface. All sequences are aligned by the -35 and Pribnow (-10) regions.

assumption in this analysis is that ORF2 and ORF3 have σ^{43} Gram positive consensus type promoters. This assumption is based simply on the fact that the 5' upstream regions of these 2 open reading frames did not contain any promoter consensus elements which have been reported as recognition sites for other Gram positive holoenzymes such as σ^{37} , σ^{32} , σ^{29} or σ^{28} (Doi and Wang, 1986). For comparison, previously identified promoters for two *C. acetobutylicum* genes are included in Fig 6.6.

Like initiation, the termination of transcription by RNA polymerase is a key event in gene expression. Prokaryotic terminators, particularly those found in *E. coli*, fall into 2 classes: factor dependent and factor independent terminators. Factor dependent terminators require the presence of rho-protein (Roberts, 1969), or of other factors (Kingston and Chamberlin, 1981) for termination to occur. The nucleotide sequence of these factor dependent terminators show little similarity amongst themselves, as well as with factor independent terminators (Holmes et al., 1983). On the other hand, factor independent terminators have been shown to be functionally active in *in vitro* assays in the absence of any protein factors other than RNA polymerase (Brendel and Trifonov, 1984).

Factor independent terminators show a region of dyad symmetry downstream of the 3' stop codon of an open reading frame. This dyad symmetry is typically rich in GC base pairs and allows for the formation of a stable hairpin structure in the RNA transcript. On average, the stem contains 77% GC base pairs and is stable with a free energy of $\Delta G = -18.0 \pm 7.3 \text{ kcal mol}^{-1}$ (Brendel and Trifonov, 1984). A typical hairpin stem has a length of 8 ± 2 base pairs and a loop of 5 ± 1 base pairs. Furthermore, just downstream of this region of dyad symmetry, there is a stretch of consecutive thymine residues. Typically, this stretch is 10 base pairs long containing at least 7 thymine residues. The region of dyad symmetry has been implicated in slowing down the polymerase, whereas the thymine rich region allows for the formation of a rU·dA hybrid that may facilitate release of the transcript (Martin and Tinoco, 1980).

Using the above criteria, sequences that could resemble factor independent terminators were searched for in the pSEQ13A insert DNA sequence immediately downstream of both ORF1 and ORF2. No structures resembling factor independent terminators were identified in these searches. As in any living system, there are exceptions to the rules (for examples see Brendel and Trifonov, 1984). Regardless of the biological implications of some of these exceptions, it does not appear that a region of dyad symmetry and an adjacent stretch of thymine residues unequivocally characterize the termination signal. These features may be enough to provide the proper conditions necessary for

termination, however they are apparently not required (Brendel and Trifonov, 1984).

The structures that characterize factor independent transcription terminators have primarily been identified from studies of genes isolated from *E. coli* and other enterobacteria (Brendel and Trifonov, 1984). With a few exceptions (Mongkolsuk et al., 1985; Pulido and Jiménez, 1987), very little has been documented about such structures in Gram positive organisms. Gram positive organisms may therefore have alternative termination systems which may be either dependent or independent of protein factors.

It would be necessary to isolate RNA from *C. acetobutylicum* in order to determine what promoters and termination signals are functioning for these open reading frames. Since the objectives of this study were to identify *C. acetobutylicum* electron transport genes that are able to activate metronidazole, such RNA studies were deemed to be beyond the scope of this project.

6.3.6 Amino acid similarity studies

The deduced amino acid sequences of the open reading frames ORF1, ORF2 and ORF3 were used to search the GenBank, EMBL, SWISS-Protein, NBRF-Nucleic and NBRF-Protein databases using the FASTA and TFASTA computer programs described by Pearson and Lipman (1988).

The best similarity to the deduced amino acid sequence from the complete open reading frame, ORF2, was obtained with the flavodoxin sequences from *Azotobacter vinelandii* (Bennett et al., 1988), *Clostridium* MP (Tanaka et al., 1974), *Anabaena variabilis* (Leonhardt and Straus, 1989) and *Klebsiella pneumoniae* (Drummond, 1985). GAP (Devereux et al., 1984), the alignment program based on the methods of Needleman and Wunsch (1970), was used to compare this deduced amino acid sequence to the above flavodoxin sequences. The amino acid sequence of the *A. vinelandii* flavodoxin had 29% identity and 55% similarity to the deduced amino acid sequence of ORF2. Likewise, the amino acid sequences of the *Clostridium* MP, *Ana. variabilis* and *K. pneumoniae* flavodoxins

1	MAIIGILGTPYNTVERSPFWWNKVSYTRQSFIDVFQELGHTVIVLPVD-K	49
IPFIIPVNTE	10
50	TENIKNYLPLVDKIVLTGGVDVSPYLYGEEP HQALGTTDFIRDRFELAAI	99
11	PEI IKAQLENV DGLILSGGHDVNPLLFNEEP HKLLGNTMNERDTFDSMLI	60
100	KAALEANKPILGVC RGLQLLN VYFGGTLYQDLSLT-SSQIKHLQSPTPQE	148
61	KYAFEMEKPILGICRG CQILNVVCGGTLYQDCSLAKDSYIKHCQGHTPAQ	110
149	VPTHHISVEKEVL-----	161
111	-PSHTIQIEKNSELYTILGEKALANSFHHMSIKDVADGFKVVAIANDEII	159

Figure 6.7 Comparison of the amino acid sequence of the 3'-truncated open reading frame which is downstream of the *L. lactis* X-prolyl dipeptidyl aminopeptidase (pepXP) gene (Nardi et al., 1991) (top strand) with the pSEQ13A 5'-truncated open reading frame (ORF1) (bottom strand). The symbol comparison table described by Gribskov and Burgess (1986) was used to calculate percentage similarity and percentage identity. The symbols between the sequences are as follows: |, represents identity; :, and • represent closer and further evolutionary distance between similar amino acids respectively.

had 29, 26 and 24% identity and 51, 55 and 56% similarity respectively. All figures referring to percent identity and percent similarity reflect data obtained from the entire length of the respective amino acid sequences. Based on the alignments to these and other flavodoxins, the fact that ORF2 has been identified as the gene responsible for class V sensitivity to metronidazole, as well as the report by Chen and Blanchard (1979) that shows reduced flavodoxin can transfer electrons to metronidazole, the sequence corresponding to ORF2 is identified as coding for a flavodoxin from *C. acetobutylicum* P262 (Santangelo et al., 1991).

No conclusive information was obtained from database searches using the amino acid sequence derived from 5'-truncated open reading frame, ORF1, as the query sequence. However, this amino acid sequence can be easily aligned with an unidentified open reading frame that is truncated at the 3' end (Fig. 6.7). This 3'-truncated unidentified open reading frame is downstream of the X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* NCDO763 (Nardi et al., 1991). The alignment, which was generated using the GAP program described above, shows a 64.5% similarity and a 51.2% identity between the two amino

```

      10      30      50      70
G GAA ATA ATG AAA ATA GTT AAT CGA ATT GGC AAG AAT ATG AGA TTA TTA ATA ATA ATG ACT TTA ATT ATT TTT GCT GTA CTA ATA ATT
E I M K I V N R I G K N M R L L I I M T L I I F A V L I I I

      90      110      130      150      170
TTA CCT CTA ATT ATT TTA AAA GGT AGT GGA TTT AGT ATT AGC AAA TTC AAT TTA GAT AAC AAA AAT TTA GTG AAA AAT TCA CAA ATA ACT
L P L I I L K G S G F S I S K F N TTA GAT AAC AAA AAT TTA GTG AAA AAT TCA CAA ATA ACT

      190      210      230      250
TTT CCG GTT AAT GGT AAG GTT AAA TTA TAC CAT AAA AAG GAT AAC TCA GTT GAA GAG TTA GAT TTG GAA GAT TAT ATA ATG GGG GTT GTA
F P V N G K V K L Y H K K D N S V E E L D L E D Y I M G V V

      270      290      310      330      350
TCA AGT GAA GTT CCA GCA AAT TTT GAG GAA GAA GCT TTA AAG GCT CAA GCT GTA GCA GCT AGA ACA TTC TAT ATG AAC AAG AGA AAT CAA
S S S E V P A N F E E A L K A Q A V A A R T F M N K R N Q

      370      390      410      430
CCT TGT AAT TAT GCA AAA GAA AAA GGT GCA GAA ATT TGC GAT ACA ACT CAT TGT CAA GTT TAT ATG AGT AAG GAA GAA AGA ATG AAA AGC
P C N Y A K E K G A E I C D T T H C Q V Y M S K E E R M K S

      450      470      490      510      530
TGG AGC AGT AGT GAA GCA AAA AGT GAT TGG GAA AAA ATT GAG AGG GCT GTT GAA GAA ACA AAA GGA CAA GTA TTA ACT TAT AAT GGA CAA
W S S E A K S D T G E K I E R A V E E T K G Q L T Y N G Q

      550      570      590      610
GTG CTC GAA TAT CAG AAT TTT TCG CTA TTA GTT CTG GAA AAA ACA GAG GAT GCT AAG GAC GTA TTT TCT GTT GAT GTA CCA TAT CTT AAA
V L E Y Q N F S L L V L E K T E D A K D V F S V D V P Y L K

      630      650      670      690      710
TCA GAA GAA AGT AAA GGT GAA GAG ATA GCG CCT AAA TAT AAA ACA TCT GTA CAG ATT GCT ACA AAT GAT TTT GTT AAT AAA ATT AAT AAC
S E E S K G E E I A P K Y K T S V Q I A T N D T V N K I N N

      730      750      770      790
AAA TAT TCA AAA GCA AAT ATA AGT AAA AGT AAT TTA GCG TCT TAT ATT AAA ATA AAA AGC TAT ACT GAA GCT GGA AGT GTA AAA GAA ATT
K Y S K A N I S K S N L A S Y I K I K S Y T E A G S V K E I

      810      830      850      870      890
CAA ATA GGA AAT GAA GTT ATA AAG GGA ACA GAA TTT AGA GAA TTA TTG GGA TTA AAT TCA ACA AAT TTC ACC TTG GAT TTT GGA ACC AAT
Q I G N E V I K G T E F R E L L G L N S T N F L D F G T N

      910      930      950      970
GCA GTA ACT GTA AAT TGT AAG GGG TAT GGT CAT GAC GTA GGA ATG AGT CAA TGG GGA GCT AAT GTA ATG GCG AAA AAT GGG GCT AAG TAT
A V T V N C K G Y G H D V G M S Q W G A N V M A K N G A K Y

      990      1010      1030      1050      1070
AGT GAA ATC CTA AAA CAT TAT TAT GTT GGA GTT GAA ATT ACT CAA ATA AAA TAT ATG CGT AAA TTA TTT GAA GTT TAT AAT TAT GTG GCA
S E I L K H Y Y V G V E I T Q I K Y M R K L F E V Y N Y V A

      1090
ATA ACG CTG AAT AAA TAA ATATGGCTTTTAA
I T L N K *

```

Figure 6.8 Nucleotide sequence of the 1109 base pair fragment of *C. acetobutylicum* insert DNA of plasmid pMET11B. The deduced amino acid sequence is given in single letter code (see Appendix D).

acid sequences, with virtually no need to introduce gaps in either of the amino acid sequences.

No conclusive information was obtained from database searches using the amino acid sequence derived from 3'-truncated open reading frame, ORF3, as the query sequence. The matches obtained from this database search were either over very short lengths of the sequence, or it was necessary to introduce frequent and lengthy gaps in order to obtain alignment.

6.3.7 pMET11B sequence

The sequence of a 1 109 base pair fragment of the *C. acetobutylicum* insert DNA of pMET11B was determined by a post-doctoral researcher in the Anaerobe Unit at the University of Cape Town. Since pMET11B was isolated in this study, the sequence of this fragment is reported here. A single open reading frame of 364 amino acids was coded for by 1 092 of the 1 109 base pairs, and was truncated at the 5' end (Fig. 6.8).

Using the deduced acid sequence (Fig. 6.8) as a query sequence to carry out database searches, it was determined that this open reading frame coded for a *spoIID* gene from *C. acetobutylicum*. Alignment of this sequence with the *B. subtilis* and *B. amyloliquefaciens* SpoIID protein sequences showed 55.8 and 52.5% similarity and 36.5 and 34.0% identity respectively. It was however determined that this 1 109 base pair fragment was not the region on pMET11B responsible for class V sensitivity towards metronidazole.

6.4 Discussion

From the exonuclease III shortened clones, it was determined that the complete open reading frame, ORF2, was the gene responsible for class V sensitivity toward metronidazole in clones pMET13A, pMET030 and pMET190. The nucleotide sequence of ORF2 has the coding capacity for a 17 763 M_r protein. Based on the deduced amino acid sequence of this open reading frame and the ability of this gene product to activate metronidazole, it has been identified as a *C. acetobutylicum* flavodoxin gene. The amino acid sequence of nine other flavodoxins has been determined by direct amino acid sequencing, or deduced from their nucleotide sequence. Wakabayashi et al. (1989) aligned seven of these flavodoxin sequences, and this alignment was used as the basis for the comparison shown in Fig. 6.9, which includes the *C. acetobutylicum* flavodoxin. The *C. acetobutylicum* flavodoxin shows some degree of similarity to the other flavodoxins. The invariant regions are located primarily at the FMN binding domains (the redox-active prosthetic group), which are included in positions 11-23, 58-64 and 101-114 (Wakabayashi et al., 1989).

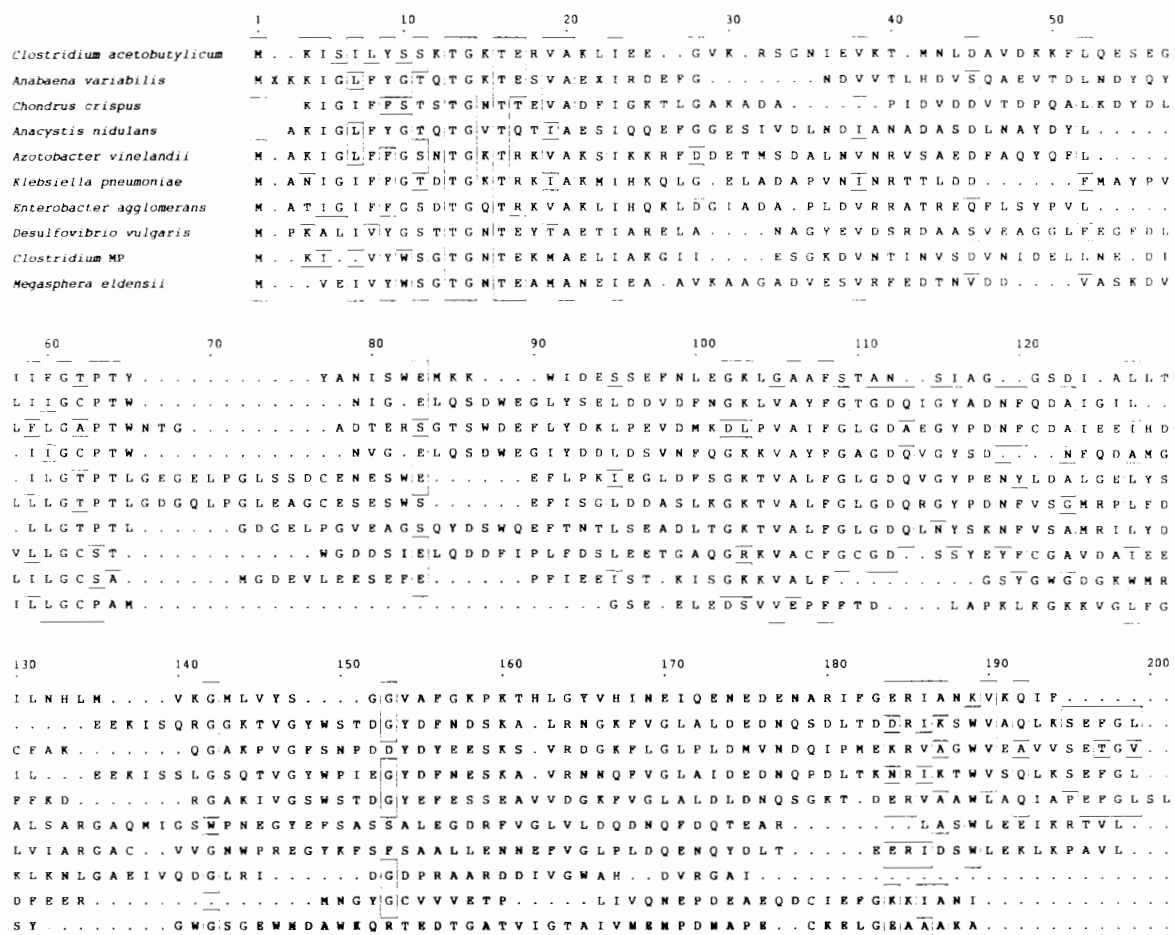


Figure 6.9 Comparison of the amino acid sequence of the *C. acetobutylicum* flavodoxin to nine other flavodoxin amino acid sequences. The flavodoxin sequences are from *A. variabilis* (Leonhardt and Straus, 1989), *Chondrus crispus* (Wakabayashi et al., 1989), *Anacystis nidulans* (Laudenbach et al., 1988), *A. vinelandii* (Bennett et al., 1988), *K. pneumoniae* (Drummond, 1985), *Enterobacter agglomerans* (W. Klingmüller, personal communication), *Desulfovibrio vulgaris* (Dubourdieu and Fox, 1977; Krey et al., 1988), *Clostridium MP* (Tanaka et al., 1974) and *Megasphaera eldensii* (Tanaka et al., 1973). The boxed regions indicate sequences with identical amino acid residues. Chemically related amino acids are not indicated.

While iron-containing ferredoxin is one of the most important components of biological electron transport chains, it can be degraded as a source of iron under iron limiting conditions (Schönheit et al., 1979; Ragsdale and Ljungdahl, 1984). Certain organisms, such as members of the genus *Clostridium*, induce the synthesis of the non-iron containing electron transfer protein flavodoxin under conditions of iron stress (Pardo et al., 1990). Ragsdale and Ljungdahl (1984) showed that when *C. formicoaceticum* was grown in a medium with a low iron content, the concentration of flavodoxin reached at least 2% of the total soluble

protein. Flavodoxins mediate electron transfer at a low redox potential between prosthetic groups of microbial proteins in an analogous fashion to ferredoxin. When the production of flavodoxin is induced (as in the clostridia) it generally replaces ferredoxin in many oxidation reduction reactions, but at a slower rate (Ragsdale and Ljungdahl, 1984). However, in other organisms such as *A. vinelandii*, flavodoxin is constitutively produced and hence has a unique biological function (Tollin and Edmondson, 1980).

In the clostridia and in other anaerobes, the principal role of flavodoxin is in the phosphoroclastic breakdown of pyruvate to acetyl-CoA. In *C. acetobutylicum* electrons derived from pyruvate reduce an iron-sulfur moiety in the enzyme pyruvate ferredoxin oxidoreductase (Meinecke et al., 1989). Under iron limiting conditions, these electrons are then used to reduce flavodoxin, which in turn is reoxidized by hydrogenase with the accompanying formation of molecular hydrogen via the actions of the hydrogenase enzyme. It has been shown that in *C. acidi-urici* reduced electron carriers such as ferredoxin and flavodoxin, are also used as the electron source in the uric acid fermentation (Valentine et al., 1962). Flavodoxin can also be oxidized and reduced by the action of the clostridial NADH- and NADPH-ferredoxin oxidoreductases (Ragsdale and Ljungdahl, 1984). In *C. pasteurianum* and *C. kluyverii*, where CO₂ fixation (essentially the reverse of pyruvate catabolism) is known to occur, reduction of ferredoxin and flavodoxin occurs by reduced pyridine nucleotides via the action of these enzymes (Jungermann et al., 1971a, 1971b; Thauer et al., 1971).

In obligate anaerobic nitrogen fixers, the endogenous donor of electrons to nitrogenase is ferredoxin or flavodoxin (Mortenson, 1964; Benemann et al., 1969; Yoch et al., 1969). *A. vinelandii* however, constitutively produces a flavodoxin to serve this function (Scherings et al., 1977). Haaker and Veeger (1977) showed that in *A. vinelandii*, reducing equivalents are produced at a site localized at the cytoplasmic membrane which is specifically mediated by the action of a NADH-flavodoxin oxidoreductase. Hence, this oxidoreductase operates at a site of low pH (ca. pH=5), where the semiquinone-hydroquinone potential is high enough to allow NADH to function as a reductant. Membrane energization in turn

brings the flavodoxin hydroquinone to a low enough potential such that it can serve as the electron donor to the nitrogenase. Like *A. vinelandii*, *K. pneumoniae* and *E. aerogenes* also have specific genes (*nifF*) for the production of a flavodoxin which is used as the electron carrier for the nitrogenase component of nitrogen fixation (Drummond, 1985; W. Klingmüller personal communication).

Other known functions for flavodoxins include the mediation of electron transfer between hydrogen and sulfite in *D. vulgaris* (Hatchikian et al., 1972). Flavodoxin has also been shown to function in the light dependent reduction of NADP⁺ by plant chloroplasts (Smillie, 1965).

All known flavodoxins have low molecular weights (14 500 to 23 000 g mol⁻¹) and are usually anionic (Tollin and Edmondson, 1980). In general, flavodoxins from non-photosynthetic bacteria have a molecular weight of approximately 15 000 (short flavodoxins), while flavodoxins from cyanobacteria and algae have a molecular weight of approximately 20 000 (long flavodoxins) (Wakabayashi et al., 1989). *A. vinelandii* flavodoxin is an exception in that it is produced by a non-photosynthetic bacteria and falls into the class of long chain flavodoxins. This flavodoxin is also unique in that it is the only flavodoxin known to contain a phosphodiester linkage (Dreef-Tromp et al., 1989; Taylor et al., 1990). Dubourdieu and Fox (1977) reported that amino acid similarity amongst the long chain flavodoxins ranged from 36 to 39% whereas amino acid similarity amongst the short chain flavodoxins ranged from 25 to 35%. The amino acid similarity of the short chain *C. acetobutylicum* flavodoxin to other flavodoxins falls within the latter range.

Flavodoxins consist of a single polypeptide chain lacking disulfide bonds (cysteine content varies from 1 to 5 residues). Flavodoxins non-covalently bind one mole of FMN, and binding studies indicate that all flavodoxins have structurally analogous coenzyme binding sites (D'Anna and Tollin, 1972). Amino acid alignments support this theory, as the ribityl phosphate binding regions of different flavodoxins have a similar primary structure. With reference to Fig 6.9, Ser-11, Thr-13 and Thr-16 hydrogen bond to the FMN phosphate,

whereas Thr-64 and Asp-176 hydrogen bond to the 4'-hydroxy group of the ribityl side chain (Wakabayashi et al., 1989).

E. coli is known to produce small amounts of ferredoxin and flavodoxin. While the biological role of ferredoxin in *E. coli* has not been completely elucidated, the role of flavodoxin has been established. Reduced flavodoxin has been shown to be involved in the conversion of pyruvate formate lyase from an inactive to an active form (Knappe et al., 1974, 1984; Conradt et al., 1984). The mechanism of the reduction of flavodoxin in *E. coli* was elucidated by Blaschkowski et al. (1982), who showed that two enzymes exist capable of carrying out this reduction. The first enzyme is the CoA acetylating pyruvate flavodoxin oxidoreductase, which is analogous to the pyruvate ferredoxin oxidoreductase more commonly associated with the phosphoroclastic breakdown of pyruvate in anaerobes. The second enzyme is an NADPH dependent oxidoreductase which these workers identified as the flavoprotein "component R" previously described by Fujii and Huennekens (1974).

If the cloned *C. acetobutylicum* flavodoxin is responsible for activating metronidazole in *E. coli* F19 [pMET13A] by direct electron transfer, reasonable amounts of this flavodoxin must exist in a reduced form. The pyruvate flavodoxin oxidoreductase and the NADPH dependent oxidoreductase are both minor proteins in *E. coli* and hence may not be present in large enough amounts to reduce significant quantities of the cloned flavodoxin. This suggests that either the cloned flavodoxin is reduced by other mechanisms, or that it is not entirely responsible for activating metronidazole. Since reduced flavodoxin is capable of converting the pyruvate formate lyase to its active form, perhaps the cloned flavodoxin merely enhances this effect. This could result in a higher conversion rate of pyruvate to acetyl CoA and formate, which may in turn lead to induction of the FDH_H enzyme (see Chapter 4). This would reduce the overall oxidation reduction potential of the cell which has been shown to cause an increase in metronidazole sensitivity in *E. coli* (Onderdonk et al., 1979).

It is interesting to note that the addition of extraneous formate (as sodium formate) to culture medium increased the sensitivity of *E. coli* strains towards metronidazole (data not shown). It was also observed that this effect was reversed by increasing the nitrate concentration in the medium. Unfortunately, these formate and nitrate experiments were carried out early on in this study in an effort to determine the ideal medium for genebank screening, and hence metronidazole sensitivity of *E. coli* F19 [pMET13A] was not tested with media containing additional formate. However, in one experiment where a calculation error resulted in a ten fold increase in the nitrate concentration of the growth medium, no change in the MIC values for *E. coli* F19 [pMET13A] was observed. This indicates that with this clone, there was essentially no formate effect to overcome. Since one of the objectives of this study was to isolate electron transport genes from *C. acetobutylicum*, the identification of this flavodoxin gene fulfills this goal. However, not enough evidence was obtained to elucidate the mechanisms involved in the activation of metronidazole.

While the structure-function relationships of ferredoxins have been studied extensively, these studies are not as advanced for the flavodoxins. Flavodoxins are among the smallest flavoproteins, and therefore represent the simplest models for studying the chemistry and biochemistry of this group of proteins (Mayhew and Ludwig, 1975). Since the production of flavodoxin in the clostridia occurs under conditions of iron limitation, the cloned *C. acetobutylicum* flavodoxin can provide a platform for studying the genetics of induction in this industrially important microorganism. Furthermore, since iron limitation is a form of stress, this is the first account of the cloning of this type of gene from *C. acetobutylicum*. Since solvent toxicity also represents a form of stress to this organism, it is exciting to note that the *spoIID* gene was isolated using the same screening method. Sporulation is a response of this organism to solvent toxicity, and although the *spoIID* gene was not directly involved in the activation of metronidazole, it may be linked to other sporulation genes present on plasmid pMET11B.

Chapter 7

General conclusions

The endospore forming Gram positive rod, *C. acetobutylicum*, has been used for many years in the industrial fermentation of carbohydrates to produce the neutral solvents acetone, butanol and ethanol. Economic pressures have placed this fermentation into the history section of general microbiology textbooks, perhaps only temporarily.

The aims of this thesis included studies on small phosphorylated molecules involved in energy metabolism and cell differentiation. One of the main themes in studies on *C. acetobutylicum* has been the regulation of cell differentiation, particularly the switch from acidogenesis to solventogenesis and the induction of sporulation. In *B. subtilis*, sporulation is induced when the concentration of GTP reaches a critically low threshold level. A change in the level of GTP has also been implicated in controlling cell differentiation of other organisms. It was therefore desired to measure the nucleotide triphosphate levels in *C. acetobutylicum* in order to determine if GTP and perhaps other NTP's, play a similar role in cell differentiation.

The separation of nucleoside triphosphates in cell extracts can be accomplished using strong anion exchange HPLC. The lack of a readily available automatic integration system for chromatographic data, along with the complexity of chromatograms associated with the separation of nucleotides in biological materials, led to the development of a manual computer-linked integration system. This system was designed to be flexible so that it could be applied to other analytical equipment that generates gaussian output curves. Advances in microprocessor technology and diode-array spectrophotometric detectors has all but made this system redundant, however it is still used for general chromatographic work in our laboratory.

A comparison of nucleoside triphosphate profiles from batch fermentations of the *C. acetobutylicum* P262 wild type, sporulation deficient (*spo-1*) and solvent deficient (*cls-1*) strains was obtained. In the wild type strain and the *spo-1* mutant, there was a sharp decrease in the nucleotide triphosphate levels just prior to the pH breakpoint and the switch from acid production to solvent production. After the switch to solvent production, the nucleotide triphosphate levels rapidly increased to reach much higher levels than were seen during acidogenesis. Exponential phase nucleotide levels in the *cls-1* mutant were comparable to those observed in the wild type and *spo-1* mutant. Unlike the wild type and *spo-1* strains the *cls-1* mutant, which does not switch to solventogenesis, did not demonstrate an increase in nucleotide levels after the cessation of cell division.

While the drop in nucleotide levels just prior to the pH break point in the two differentiating *C. acetobutylicum* strains does not categorically demonstrate their role in the switch to solventogenesis and hence the induction of sporulation, this observation does coincide with previous results on cell differentiation in other organisms. The effect of nucleotide synthesis inhibitors, which decrease nucleotide triphosphate levels, add further support to the role of nucleotide triphosphates (particularly GTP) in differentiation of *C. acetobutylicum*. It is interesting to note that in the wild type strain, these nucleotide synthesis inhibitors only increase the spore titre when they were added to the fermentation medium prior to the switch to solventogenesis.

ATP is the primary currency by which a cell passes energy from one metabolic operation to another. In the wild type and *spo-1* mutant, ATP levels during acidogenesis were much lower than during solventogenesis. These results are somewhat surprising since the production of acids generates more ATP per mole of glucose than does the production of solvents. These low acidogenic phase ATP levels could perhaps be accounted for by considering that ATP must be expended in order to maintain a constant pH gradient across the cell membrane. Furthermore, the ATP calculations were based on cell number and the cessation of rapid cell division could alter the perception of the total ATP levels.

In analyzing the results of the nucleotide profile studies, it must be kept in mind that these levels represent a value for the "pool" quantity of these molecules. No conclusions can therefore be drawn on the actual turnover rates of these molecules. These difficult to interpret results merely indicate a general trend of what may be happening to the levels of these phosphorylated molecules with respect to the changing metabolism of *C. acetobutylicum*.

In addition to these nucleotide studies, an initial investigation into the genetics and molecular biology of *C. acetobutylicum* electron transport genes was performed. The antibacterial agent metronidazole played a pivotal role in the development of a negative selection system for the isolation of these electron transport genes. Metronidazole is a prodrug that needs to be reductively activated. The reduced intermediates of metronidazole cause DNA damage and hence cell death. It has been shown that *E. coli* strains with lesions in their DNA repair systems are more susceptible to metronidazole than their wild type parents. However, if these DNA repair deficient strains also lacked the ability to reduce chlorates and nitrates, they were no more susceptible to metronidazole than the wild type strains. The lack of availability of nitrate and chlorate reductase, DNA repair deficient mutants of *E. coli* led to the construction of *E. coli* F19 via transposon mutagenesis with *TnphoA*. This strain has a diminished ability to reduce *p*-nitrobenzoic acid, while retaining the *recA* mutation of its parent strain CC118. These characteristics make *E. coli* F19 an ideal candidate for the isolation of *C. acetobutylicum* electron transport genes able to activate metronidazole. The *recA* mutation of F19 renders it highly susceptible to the reduced toxic intermediates of metronidazole, while the nitroreductase deficiency renders F19 more resistant to the prodrug. *E. coli* F19 forms the basis of this negative selection system where *C. acetobutylicum* electron transport genes were cloned on recombinant plasmids that are able to activate metronidazole and overcome the nitroreductase deficiency of this strain.

Since *E. coli* F19 was developed to be used as a cloning host, and not for physiological studies of the effects of metronidazole on *E. coli*, no efforts were made to further characterize the mutation induced by *TnphoA*. However, it

would be interesting for future work to develop along these lines and hence to determine the exact nature of this mutation which renders this *E. coli* strain less susceptible to metronidazole.

Using *E. coli* F19 as a cloning host to screen a *C. acetobutylicum* gene library, 25 different *C. acetobutylicum* chromosomal DNA fragments on recombinant plasmids which activated metronidazole were isolated. These clones were classified into five groups (I-V) based on their sensitivity to metronidazole. With respect to metronidazole susceptibility, class I isolates were the least sensitive group, whereas class V isolates were the most sensitive group. After testing these 25 clones for nitroreductase, hydrogenase and pyruvate-Fd-oxidoreductase activities, it was determined that only one clone expressed weak hydrogenase activity. No positive activity was found for either nitroreductase or pyruvate-Fd-oxidoreductase in any of the clones. The hydrogenase positive clone, pMET15B2, was a class I isolate. Assuming that the metronidazole sensitive phenotype is due to the hydrogenase activity demonstrated by this clone, the relatively low levels of hydrogenase activity may account for the class I susceptibility grouping.

Although the hydrogenase enzyme plays a critical role in electron flow in *C. acetobutylicum*, it was desired to further classify all 25 isolates and to determine what *C. acetobutylicum* genes were responsible for class V susceptibility to metronidazole. Therefore, no further effort was made to specifically characterize the hydrogenase positive clone pMET15B2. Due to the possible extent of work involved in the characterization of this clone, it was determined that this should form the basis of a separate research project stemming from these initial studies.

Using DNA hybridization and restriction endonuclease mapping techniques, it was revealed that four of the metronidazole activating *C. acetobutylicum* insert DNA fragments on recombinant plasmids were linked in an 11.1 kb chromosomal fragment. Three of these clones, pMET13A, pMET030 and pMET190, were class V isolates, whereas the fourth clone was the hydrogenase positive isolate pMET15B2. Therefore, the 11.1 kb chromosomal fragment contained at least two regions responsible for activating metronidazole. The

region responsible for class V sensitivity to metronidazole was localized to a 2 kb *EcoRI-EcoRV* fragment present in each of the clones pMET13A, pMET030 and pMET190.

The nucleotide sequence of this 2 kb region was determined and a gene coding for a flavodoxin-like protein was identified. Although direct electron transfer from reduced electron carriers has been implicated in the activation of metronidazole, recent evidence strongly suggests that metronidazole is reduced via enzymatic routes. It would be interesting to identify how this *C. acetobutylicum* flavodoxin protein is activating metronidazole in *E. coli* F19. There is a strong possibility that the overproduction of flavodoxin in *E. coli* alters the anaerobic metabolic pathways such that the redox environment is lowered. Hence the flavodoxin itself may not be responsible for reducing metronidazole, but rather may be activating low redox potential enzymes in *E. coli* capable of reducing metronidazole.

Much work can continue from this initial study, and indeed some research projects are already underway in our laboratory. Apart from the characterization of the hydrogenase positive clone pMET15B2, the two remaining class V isolates, pMET11B and pMET13C1, are being characterized using molecular genetic techniques. PAGE studies of cell extracts of the 25 isolates revealed that 3 clones, pMET7A, pMET7C and pMET14B, had a visible extra protein product. Furthermore, 2 of these clones, pMET7C and pMET14B, cross hybridized with clones pMET140 and pMET2D. Molecular genetic characterization of all 5 of these clones is also underway.

It was never intended to study the mode of action of metronidazole, and therefore this drug was primarily used as a tool for the isolation of *C. acetobutylicum* electron transport genes. However, the medical importance of this drug warrants further investigation into how these cloned *C. acetobutylicum* genes may be involved in metronidazole activation. Another advantage of this system is that it provides the capability to test novel analogs of metronidazole for their mutagenic activity. A *recA*⁺ revertant of *E. coli* F19 has been isolated, and in

conjunction with the cloned metronidazole activating genes, we are able to easily determine if an analog of metronidazole needs to be reductively activated and if this active product causes DNA damage. Since the DNA is the site of action for metronidazole, it is inherently mutagenic. Medically, it is desirable to obtain a similar drug that needs to be reductively activated, yet has a different site of activity. Indeed we have already used this system to test 5 novel compounds, which are related to metronidazole, and initial evidence suggest that one of these compounds may not act upon DNA.

Electron transport systems in *C. acetobutylicum* play a pivotal role in determining the carbon flow during the fermentation of carbohydrates. Characterization of these electron transport genes may eventually lead to knowledge that will allow for enhancement of the productivity of the ABE fermentation. In view of recent developments concerning the genetic manipulation of *C. acetobutylicum*, it is likely that some electron transport genes will be specific targets for mutation aimed at improving *C. acetobutylicum* strains.

Appendix A

Media, buffers and solutions

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Appendix A

Media, buffers and solutions

Media, buffers and solutions were sterilized by autoclaving at 121 °C for 20 min when required. Heat labile substances were sterilized by filtration through 0.22 µm membrane filters (Millipore).

A.1 Media

The methods of Moore (1966) and Hungate (1969) were used to prepare pre-reduced, oxygen-free media. Most of the oxygen was driven off by gentle boiling of media for 15-20 min. Aliquots (10 ml) of the warm medium were dispensed into Hungate tubes and perfused with H₂ and CO₂ prior to sterilization by autoclaving. Large volume culture media were not perfused with H₂ and CO₂, but rather autoclaved and transferred to an anaerobic atmosphere in the anaerobic glove box at least 15 h prior to inoculation. Media containing agar was autoclaved, poured into petri dishes and stored in anaerobic jars (flushed with H₂ and CO₂ for 20 min), with silica gel in muslin bags. Cysteine hydrochloride was added to most anaerobic media to scavenge any residual oxygen.

A.1.1 *Clostridium acetobutylicum* minimal medium (CAMM) (Long et al., 1983)

Ingredient	Amount/l
Glucose	50.0 g
Ammonium nitrate	7.2 g
Calcium carbonate	20.0 g
Salts stock solution ^a	40.0 ml
MgSO ₄ ·7H ₂ O (20% w/v)	1.0 ml
MnSO ₄ ·4H ₂ O (1% w/v)	1.0 ml
FeSO ₄ ·7H ₂ O (1% w/v)	1.0 ml
ZnSO ₄ ·7H ₂ O (5% w/v)	1.0 ml
NaMoO ₄ ·2H ₂ O (1% w/v)	1.0 ml
<i>p</i> -Aminobenzoic acid (0.1% w/v)	1.0 ml
Thiamine HCl (0.1% w/v)	1.0 ml
Biotin (0.02% w/v)	250.0 µl
Cysteine HCl	0.5 g

Sterile potassium phosphate buffer (1.0 M), pH 7.0, was added to the autoclaved broth at a final concentration of 0.05 M.

^a Salts stock solution contained/l:

CaCl ₂	0.2 g
MgSO ₄ ·7H ₂ O	0.2 g
K ₂ HPO ₄	1.0 g
KH ₂ PO ₄	1.0 g
NaHCO ₃	10.0 g
NaCl	2.0 g

A.1.2 *Clostridium* basal medium (CBM) (O'Brien and Morris, 1971)

Ingredient	Amount/l
Glucose	10.0 g
Casein hydrolysate	4.0 g
Yeast extract	4.0 g
MgSO ₄ ·7H ₂ O (20% w/v)	1.0 ml
MnSO ₄ ·4H ₂ O (1% w/v)	1.0 ml
FeSO ₄ ·7H ₂ O (1% w/v)	1.0 ml
<i>p</i> -Aminobenzoic acid (0.1% w/v)	1.0 ml
Thiamine HCl (0.1% w/v)	1.0 ml
Biotin (0.0002% w/v)	250.0 µl
NaHCO ₃	1.0 g
Cysteine HCl	0.5 g
Resazurin stock solution (0.02% w/v)	10.0 ml

The required percentage agar (1.5% w/v) was added to CBM medium made as above. Cysteine HCl (10.0 ml) and NaHCO₃ (20 ml) were added as sterile stock solutions after the medium had been autoclaved and prior to pouring. The solid medium did not contain the anaerobic indicator, resazurin.

A.1.3 Luria-Bertani medium (LB)

Ingredient	Amount/l
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Agar (solid medium only)	15.0 g

A.1.4 2x YT medium

Ingredient	Amount/l
Tryptone	16.0 g
Yeast extract	10.0 g
NaCl	5.0 g
Agar (solid medium only)	15.0 g

A.1.5 *Escherichia coli* anaerobic medium (ECAM)

Ingredient	Amount/l
Glucose	10.0 g
Tryptone	10.0 g
Yeast extract	1.0 g
NaCl	5.0 g
NaHCO ₃	840.0 mg
Tris-base	12.1 g
Agar (solid medium only)	15.0 g

The pH of this media was adjusted to 8.0 with HCl prior to autoclaving.

A.2 Media additives

Media were cooled to 50°C before addition of antibiotics, XGal, XP or IPTG.

Plates containing these additives were stored for no longer than one week at 4°C.

A.2.1 Antibiotics

Antibiotic stock solutions were as follows:

Ampicillin	100 mg/ml water
Chloramphenicol	20 mg/ml ethanol (96%)
Kanamycin	62.5 mg/ml water
Tetracycline	12.5 mg/ml ethanol (50%)

All antibiotics were filter sterilized and stored at -20°C, except for tetracycline which was always made fresh

A.2.2 IPTG (isopropyl-b-D-thio-galactopyranoside)

IPTG (100 mM)	23.4 mg
Distilled water	1.0 ml

This solution was stored in aliquots at -70°C.

A.2.3 XGal (5-bromo-4-chloro-3-indolyl-b-galactoside)

XGal (2% w/v)	0.2 g
Dimethylformamide	10.0 ml

This solution was stored at -70°C.

A.2.4 XP (5-bromo-4-chloro-3-indolyl phosphate)

XP	80.0 mg
Dimethylsulfoxide	1.0 ml

This solution was stored at -70°C.

A.3 Buffers and solutions**A.3.1 Agarose gel electrophoresis solutions****A.3.1.1 DNA loading solution (6x)**

Bromophenol blue	250.0 mg
Sucrose	40.0 g
Distilled water	to 100.0 ml

This solution was stored at 4°C.

A.3.1.2 Tris acetate buffer (50x)

Tris-base	242.0 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100.0 ml
Distilled water	to 1000.0 ml

A.3.1.3 Tris borate buffer (5x)

Tris-base	54.0 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20.0 ml
Distilled water	to 1000.0 ml

A.3.2 Biuret solution for protein determination

CuSO ₄ ·5H ₂ O	1.5 g
NaKC ₄ H ₄ O ₆ ·4H ₂ O (NaK-tartrate)	6.0 g
Distilled water	to 500.0 ml

With constant stirring, add 300 ml of 10% (w/v) NaOH. Make up to 1 liter with H₂O. Solution is an intense blue color and must be stored in a dark glass bottle to protect from light.

A.3.3 DNA hybridization solutions**A.3.3.1 Alkali fixation solution**

NaOH (0.4 M)	16.0 g
Distilled water	to 1000.0 ml

A.3.3.2 Denaturing solution

NaCl (1.5 M)	87.6 g
NaOH (0.5 M)	20.0 g
Distilled water	to 1000.0 ml

A.3.3.3 Denhardt's solution (50x)

Bovine serum albumin (1% w/v)	1.0 g
Ficoll (1% w/v)	1.0 g
Polyvinylpyrrolidone-40 (1% w/v)	1.0 g
Distilled water	to 100.0 ml

This solution was stored in aliquots (10 ml each) at -20 °C.

A.3.3.4 Hydrolysing solution

HCl, concentrated (0.25 M)	26.0 ml
Distilled water	to 1000.0 ml

A.3.3.5 Neutralizing solution

EDTA (1.0 mM)	372.0 mg
NaCl (1.5 M)	87.6 g
Tris-base (0.5 M)	60.5 g
Distilled water	to 1000.0 ml

This solution was adjusted to pH 7.2 with HCl.

A.3.3.6 Salmon sperm DNA

A 10 mg/ml solution was made in TE buffer. The DNA solution was sonicated at full power (20 microns) for 10 min in a MSE Soniprep sonicator. The solution was aliquoted and stored at -20°C. Immediately before use the DNA was denatured by boiling for 10 min followed by cooling on ice.

A.3.3.7 SSC (20x)

NaCl (3.0 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1000.0 ml

This solution was adjusted to pH 7.0 with NaOH and autoclaved.

A.3.4 Exo-nuclease III shortening solutions**A.3.4.1 Exo buffer**

Tris-HCl buffer (1 M, pH 8.0)	660.0 µl
MgCl ₂ (0.1 M solution)	66.4 µl
Distilled water	9.3 ml

A.3.4.2 Klenow mixture

Tris-HCl buffer (1 M, pH 8.0)	3.0 µl
MgCl ₂ (1 M solution)	6.0 µl
Distilled water	20.0 µl

One µl of klenow was added per µg of DNA just prior to use.

A.3.4.3 Ligase mixture

Ligation buffer (10x)	144.0 µl
T4 ligase (1 U/µl)	24.0 µl
Distilled water (sterile)	1282.0 µl

A.3.4.4 S₁ buffer (10x)

KOAc (3 M, pH 4.6 solution)	1.1 ml
NaCl (5 M solution)	5.0 ml
Glycerol	5.0 ml
ZnSO ₄ 30.0 mg	

A.3.4.5 S₁ mixture

S ₁ buffer (10x)	41.0 µl
S ₁ nuclease (40 U/µl)	1.5 µl
Distilled water (sterile)	259.0 µl

A.3.4.6 S₁ stop

EDTA (0.05 M)	372.2 mg
Tris-base (0.3 M)	726.6 mg
Distilled water (sterile)	20.0 ml

The pH of this solution was not adjusted as alkaline conditions are required to inactivate the S₁ nuclease.

A.3.5 General DNA manipulation solutions**A.3.5.1 ATP (10x)**

Adenosine 5'-triphosphate	30.0 mg
Distilled water (sterile)	5.0 ml

This solution was adjusted pH to 7.0, and stored in 100 µl aliquots at -70 °C.

A.3.5.2 DTT (1M)

Dithiothreitol	3.1 g
Sodium acetate (10 mM, pH 5.2 solution)	20.0 ml

This solution was filtered and stored in aliquots at -20 °C

A.3.5.3 EDTA (0.5M, pH 8.0)

EDTA	168.1 g
Distilled water	to 1000.0 ml

The EDTA did not dissolve until the pH was raised to 8.0 with approximately 20 g of solid NaOH. This was autoclaved and stored at room temperature.

A.3.5.4 Ethidium bromide solution

(2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide). A solution of 10 mg/ml was made in distilled water and stored in a dark bottle.

A.3.5.5 Isopropanol (salt saturated)

Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

A.3.5.6 Klenow (DNA polymerase I) buffer

The buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 7.6)	0.1 M	1.0 ml
MgCl ₂ (1 M)	0.1 M	1.0 ml
NaCl (5 M)	0.5 M	1.0 ml
2-mercaptoethanol	0.7 M	50.0 µl
Distilled water		7.0 ml

A.3.5.7 Ligase dilution buffer

The buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 7.6)	20 mM	0.2 ml
EDTA (0.5 M, pH 8.0)	1 mM	2.0 µl
DTT (0.5 M)	5 mM	10.0 µl
KCl (1 M)	60 mM	0.6 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water		4.8 ml

A.3.5.8 Ligation buffer (10x)

The buffer was made according to the following table and stored at -70°C.

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 7.6)	66 mM	660.0 µl
MgCl ₂ (1 M)	6 mM	66.0 µl
ATP (0.1 M)	1 mM	100.0 µl
DTT	100 mM	15.4 mg
Distilled water		174.0 µl

A.3.5.9 Phenol (TE saturated)

Phenol (200 g) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10x) or until the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1x) at -20°C.

A.3.5.10 Restriction enzyme buffers

Stock solution	Final conc.
Tris-HCl buffer (1 M, pH 7.9)	0.1 M
MgCl ₂ (1 M)	0.1 M
DTT (0.5 M)	10.0 mM
BSA (10 mg/ml)	1 mg/ml
Glycerol	44% (v/v)
NaCl (5 M)	0, 50, 100 or 150 mM

These restriction enzyme buffers were made according to the following table and stored at -20°C.

Stock solution	Salt concentration (mM)			
	0	50	100	150
Tris-HCl (1 M, pH 7.9)	1 ml	1 ml	1 ml	1 ml
MgCl ₂ (1 M)	1 ml	1 ml	1 ml	1 ml
DTT (0.5 M)	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA (10 mg/ml)	1 ml	1 ml	1 ml	1 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml
NaCl (5 M)	-	1 ml	2 ml	87.7 mg
Distilled water	2.4 ml	1.4 ml	0.4 ml	2.4 ml

A.3.5.11 Restriction enzyme dilution buffer

This buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 7.5)	10 mM	0.1 ml
KCl (1 M)	50 mM	0.1 ml
Distilled water		5.3 ml
Filter sterilize this solution and then add the following constituents:		
2-mercaptoethanol	10 mM	7.0 µl
Gelatin (10 mg/ml)	100 µg/ml	0.1 ml
Glycerol	44% (v/v)	4.4 ml

A.3.5.12 *Sma*I restriction endonuclease buffer

This buffer was made according to the following table and stored at -20°C.

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 8.0)	0.1 M	1.0 ml
KCl (1 M)	0.2 M	2.0 ml
MgCl ₂ (1 M)	0.1 M	1.0 ml
DTT (0.5 M)	10 mM	0.2 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water		1.4 ml

A.3.5.13 Tris-EDTA (TE) buffer (100x)

Component	Amount
Tris-base	121.1 g
EDTA (0.5 M, pH 8.0 solution)	200.0 ml
Distilled water	to 1000.0 ml

Adjust to desired pH (eg. 7.5, 7.6 or 8.0) with HCl and autoclave. Dilute with sterile water before use.

A.3.6 Plasmid preparation solutions**A.3.6.1 Solution I (10x)**

Component	Final conc.	Amount
Glucose (anhydrous)	500 mM	9.01 g
Tris-base	250 mM	3.03 g
EDTA (disodium salt)	100 mM	3.72 g
Distilled water		to 100.00 ml

Adjust to pH 8.0 with HCl and autoclave. Dilute with sterile water before use.

A.3.6.2 Solution II

This solution was prepared fresh on a weekly basis according to the following table:

Component	Final conc.	Amount
NaOH (10 N solution)	0.2 N	0.5 ml
SDS (10% w/v solution)	1.0 %	2.5 ml
Distilled water		22.0 ml

A.3.6.3 Solution III

Component	Amount
K-acetate	147.0 g
Acetic acid (glacial)	pH to 4.8
Distilled water	to 500.0 ml

The potassium acetate was dissolved in as little water as possible, and the pH was adjusted to between 4.8 and 5.0 with acetic acid before bringing the volume up to 500 ml.

A.3.7 Polyacrylamide gel electrophoresis (PAGE) solutions

A.3.7.1 Acrylamide solution

Acrylamide	29.2 g
Bis-acrylamide	0.8 g
Distilled water	to 100.0 ml

This solution was filtered through Whatman's No.1 paper and stored in a dark bottle at 4°C.

A.3.7.2 Ammonium persulphate solution

A 10% (w/v) ammonium persulphate solution was freshly prepared as needed.

A.3.7.3 Coomassie blue staining solution

Coomassie blue R250	2.5 g
Destain solution (see below)	to 500.0 ml

This solution was stirred overnight to ensure complete dissolving of the dye. The following day, it was filtered through Whatman's No.1 paper.

A.3.7.4 Destain solution

Acetic acid (glacial)	250.0 ml
Methanol	750.0 ml
Distilled water	1.5 l

A.3.7.5 Reservoir buffer

Tris-base	15.15 g
Glycine	72.05 g
SDS (10% w/v solution)	50.00 ml
Distilled water	to 5.00 l

The pH of this solution should be approximately 8.5.

A.3.7.6 Resolving gel buffer

Tris-base	18.17 g
Distilled water	to 100.00 ml

The pH of this solution was adjusted to 6.8 with HCl.

A.3.7.7 Sample treatment buffer

Stacking gel buffer (see below)	2.5 ml
SDS (10% w/v solution)	4.0 ml
Glycerol	2.0 ml
2-mercaptoethanol	1.0 ml
Distilled water	0.5 ml

This solution was stored in aliquots at -20°C.

A.3.7.8 SDS solution

A 10% (w/v) solution of SDS was prepared as required.

A.3.7.9 Stacking gel buffer

Tris-base	6.06 g
Distilled water	to 100.00 ml

The pH of this solution was adjusted to 6.8 with HCl.

Appendix B

General techniques

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Appendix B

General techniques

B.1 Plasmid preparations

B.1.1 Small scale (miniprep)

Plasmid was isolated from a 5 ml overnight culture (LB + Ap, 100 µg/ml) as described by Ish-Horowicz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 200 µl Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 400 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The sample was vortexed briefly and placed on ice for 5 min, before the addition of 300 µl ice-cold Solution III (5 M KOAc, pH 4.8). The sample was vortexed briefly, and, after 5 min on ice, cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (750 µl) was removed to a fresh tube and sedimented by centrifugation with an equal volume of isopropanol for 5 min. The pellet was resuspended in TE (600 µl), NaClO₄ was added (60 µl, 5 M), and the DNA was sedimented with an equal volume of isopropanol, washed with 70% ethanol, air dried and resuspended in 50 µl TE buffer.

B.1.2 Large scale (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 6 000 g for 5 min and then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 12 000 g for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 27 000 g for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopicnic CsCl-EtBr ultracentrifugation (Maniatis et al. 1982). The plasmid preparation was prepared

for ultracentrifugation by the addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 27 000 g for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.2 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated from the CsCl solution by the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10 µl (diluted in TE) between 220 and 310 nm. The concentration was determined by using the relationship $A_{260} = 1$ for 50 µg/ml double-stranded DNA.

B.2 Restriction endonuclease digestion

Restriction digests were carried out using one of the four restriction buffers (Appendix A) according to the salt requirements of the particular enzyme. The enzyme *SmaI* required a unique buffer (Appendix A). Digestion volumes were routinely 20 µl containing 300-500 ng DNA and one unit of restriction enzyme. Digestions were done at 37°C (most enzymes) for 1 h. Concentrated enzyme stocks were diluted to 1 or 2 units using universal restriction enzyme dilution buffer (Appendix A). For electrophoretic analysis, the digestions were terminated by the addition of 5 µl DNA loading solution (Appendix A) to the 20 µl digestions. If the sample was to be used for ligation the digestion was terminated by a phenol-chloroform extraction. The DNA solution was extracted with the addition of phenol (1/10 volume, TE-saturated) and an equal volume of chloroform:isoamyl alcohol (24:1). The mixture was vortexed briefly, and the two phases were separated by centrifugation. The aqueous phase was extracted twice with water-saturated ether. The DNA was precipitated by the addition of

5M NaClO₄ (1/10 volume), an equal volume of isopropanol, and 15 min centrifugation. If the DNA concentration was less than 2 µg/100µl *E. coli* tRNA was added (2 µg) before precipitation. After centrifugation the pellet was washed with 70% ethanol and resuspended in TE buffer.

B.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-acetate buffer (Appendix A) was routinely used, however Tris-borate buffer (Appendix A) was occasionally used. Sigma type II agarose was used at varying concentrations. The amount of DNA loaded/lane also varied with the sizes and number of fragments but under normal circumstances about 300 ng of plasmid DNA was used. The gels were electrophoresed at 2 V/cm for 16 h. Gels were stained in electrophoresis buffer containing EtBr (0.5 µg/ml) for 15-30 min. DNA bands were visualised using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f 4.7 was used.

B.4 DNA ligation reactions

DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions, for example in subcloning (use 5 pmole DNA/ml). DNA concentration was calculated using the formula 1 pmole = (0.662 × kb)µg.

Ligation reactions containing DNA, ligation buffer (Appendix A) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15°C overnight using

0.1-0.25 U of ligase, whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100 x more ligase.

B.5 Subcloning protocol

The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (1%) (Seaplaque) in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70°C for 5 min in a microfuge tube and the required amounts (2 µl vector DNA, 8 µl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ligase and water (10 µl). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70°C for 5 min, and then diluted with 4 volumes of TE buffer (Appendix A).

B.6 The preparation and transformation of competent *E. coli* cells

Competent cells of *E. coli* were prepared essentially as described by Dagert and Ehrlich (1979). An overnight culture of *E. coli* was diluted 1:1 000 into 50 ml pre-warmed (37°C) YT medium and grown with shaking to early exponential phase ($OD_{600} = 0.3-0.6$) (2-4 h). The culture was cooled on ice for 5 min, and the cells were collected by centrifugation at 5 000 x g. The cell pellet was washed once with 50 ml of ice cold 0.1 M $MgCl_2$, and resuspended in 25 ml of ice cold 0.1 M $CaCl_2$. After 1 h on ice the cells were collected by centrifugation and resuspended in 5 ml of 0.1 M $CaCl_2$. The competent cells were kept on ice for at least 1 h before use or aged overnight at 4°C to improve their competency. For long term storage, competent cells were treated with glycerol to a final concentration of 15% (v/v) and the cells frozen at -70°C. Competent cells prepared in this way were suitable for use for more than 1 year.

Plasmid DNA (1-5 ng) or a fraction of a ligation reaction (20-100 ng DNA) was added to 100 μ l of competent cells on ice. After 10 min the cells were induced to take up the DNA by heat shocking the transformation mix at 42°C for 5 min. One ml of LB medium was added and the transformation mix left at 42°C for a further 30 min to allow expression of the plasmid borne antibiotic marker. Controls included: competent cells with no DNA added; unrestricted plasmid to monitor transformation frequency and linearised and ligated plasmid to monitor ligation efficiency. The transformation frequency was in the order of 3 000 to 8 000 colonies per nanogram of unrestricted plasmid DNA.

B.7 Nucleotide sequencing

B.7.1 Primer annealing reaction

The supercoiled DNA (6-10 μ g, in TE buffer) was diluted to a final volume of 20 μ l in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 μ l of 3 M sodium acetate (pH 5.2), 25 μ l of distilled water and 150 μ l of ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 μ l of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 μ l of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM MgCl₂; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 15 min at 40°C immediately prior to sequencing. The primers used were the forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 reverse sequencing primer (Amersham).

B.7.2 Sequencing reactions

DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a "Sequenase" sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [α -³⁵S]dATP (1200 Ci/mmol; Amersham).

B.7.3 Gel electrophoresis and autoradiography

The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2mm thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S -labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

B.8 Radioactive labelling of DNA probes

DNA probes were labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ to high specific activity by nick-translation (Rigby et al. 1977). The reagents were obtained in kit form (Amersham) and used according to the suppliers specifications. Contaminating nucleotides were removed from the radioactively labelled probe preparation using a Sephadex G50 spin column as described by Maniatis et al. (1982). Radioactively labelled probes were stored in lead containers at -20°C . Probes were denatured by boiling (5 min) in a fume hood just before use.

B.9 DNA hybridization

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond-N⁺ hybridization membrane (Amersham) according to the manufacturer's instructions. After staining the agarose gel with ethidium bromide, and obtaining a polaroid picture, the gel was soaked in 0.25 M HCl for 30 min to partially hydrolyse the DNA by acid depurination. After rinsing briefly in distilled water the gel was soaked in denaturing solution (Appendix A) for 30 min. The gel was again rinsed in distilled water followed by a 30 min soak in neutralizing solution (Appendix A). The gel was placed on a glass surface and a pre-wet (with neutralizing solution) piece of Hybond-N⁺ membrane was placed on top of the gel. After removal of all air bubbles between the gel and the membrane, three sheets of Whatman 3 MM filter paper, wetted in neutralizing

solution, were laid onto the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue for 60 to 90 min. After transfer, the membrane was placed on a pad of absorbent filter paper which had been soaked in 0.4 M NaOH to fix the DNA to the membrane. The membrane was rinsed briefly in 5x SSC (Appendix A).

Hybridization and washing conditions were essentially according to Maniatis et al. (1982). The membrane was gently shaken in pre-hybridization solution (Appendix A) for 4 h at 65°C, while the probe was being prepared. The radioactively-labelled probe to be used was denatured by boiling for 10 min and was added to the pre-hybridization fluid. Hybridization was carried out at 65°C overnight. The membrane was washed in 1 x SSC at 65°C, for 30 min, and after checking the radioactivity by means of a Geiger-counter, the washing was terminated and the membrane sealed in a plastic bag. The membrane was exposed to autoradiographic film (XAR-5) overnight at -70°C.

B.10 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was done according to the method of Laemmli (1970), using a Hoefer SE600 vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The 1.5 mm thick gel spacers were used. The resolving gel was prepared and degassed before pouring. Isopropanol was layered on the gel to promote a sharp interface. After the gel had polymerized (30 min), the isopropanol was removed by rinsing with the stacking gel buffer, and the stacking gel was cast.

Samples were prepared in sample treatment buffer (Appendix A) and placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant current)/gel until the dye front migrated to the end of the gel (four to five hours).

After electrophoresis the gels were stained for 3 h in coomassie blue staining solution with gentle agitation, destained and dried.

The acrylamide gels (10%) were prepared according to the following table:

Solution	Resolving gel	Stacking gel
Acrylamide solution	13.3 ml	2.6 ml
Resolving gel buffer	10.0 ml	-
Stacking gel buffer	-	5.0 ml
SDS (10%)	0.4 ml	0.2 ml
Distilled water	15.0 ml	12.2 ml
Ammonium persulphate	0.3 ml	0.1 ml
TEMED	0.1 ml	40.0 μ l

B.11 Determination of protein concentrations

Protein concentrations in solutions were determined by the biuret method as described by Gornall et al. (1949). Assays were performed in duplicate using new disposable test tubes (13 X 175 mm). The reaction contained protein solution (1.5 ml) and biuret reagent (1.5 ml) (Appendix A). After 15 min at 37°C, the absorbance of the reaction was spectrophotometrically monitored at 540 nm. Protein concentrations were calculated using a standard curve (BSA Fraction V; 0.05 to 2.5 mg ml⁻¹). Protein samples were diluted such that the absorbance did not 0.3 (approximately 2 mg protein), as this corresponds to the upper limit for linearity for this method.

B.12 Fermentation end product determination

Acetate, butyrate, acetone, butanol and ethanol were measured using a Hewlett Packard 5880A gas chromatograph fitted with a flame ionisation detector. A glass column (1.86 m X 4 mm) packed with 10% diethylene glycol adipate on 80-100 mesh chromosorb-W-HP (Supelco) was used with propanol as an internal standard. The column temperature was a linear gradient from 60 to 180°C at 20°C per min. The detector temperature was 300°C and the injector temperature was 250°C. The carrier gas was nitrogen at a flow rate of 30 ml min⁻¹.

Appendix C

E. coli strains, genotypes, and references

<i>E. coli</i> strain	Genotype/description	Reference/origin
AB1157	<i>F⁻ thr leu his pro arg lac gal ara xyl mtl str</i>	DeWit and Adelberg (1962)
AB1884	<i>uvrC34</i> (AB1157 derivative)	Howard-Flanders et al. (1966)
AB1885	<i>uvrB5</i> (AB1157 derivative)	Howard-Flanders et al. (1966)
AB1886	<i>uvrA6</i> (AB1157 derivative)	Howard-Flanders et al. (1966)
C600	<i>supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21</i>	Appleyard (1954)
CC118	<i>araD139 Δ(ara, leu)7697 ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argE_{am} recA1</i>	Manoil and Beckwith (1985)
CSH50	<i>ara Δ(lac pro) strA thi⁻ (φ80dlac⁺)</i>	Miller (1972)
CSR603	<i>recA1 uvrA6 phr1</i> (AB1886 derivative)	Sancar and Rupert (1978)
ET8051	<i>rbs lacZ::IS1 gyrA Δ(glnA-rha)VIII</i>	Pahel and Tyler (1979)
F19	this study -- nitroreductase deficient (CC118 derivative)	Santangelo et al. (1991)
HB101	<i>leuB6 trp-38 met-70 recA13 supE44 rpsL20 hsdS20(r_B⁻, r_B⁻) ara-14 proA2 lacY1 galK2</i>	Boyer and Roulland-Dussoix (1969)
JA221	<i>recA1 leuB6 trp ΔE5 hsdR⁻ hsdM⁺ lacY1</i> (C600 derivative)	Beggs (1978)
JM103	<i>endA hsdR supE sbcB thi1 strA Δ(lac proAB) λ⁻</i>	Yanisch-Peron et al. (1985)
K514	<i>thr-1 leuB6 thi-1 supE44 lacY1 tonA21 r_K⁻, m_K⁺</i> (C600 derivative)	Wood (1966)
LK111	<i>lacI_q lacZΔM15 lacY⁺</i> (K514 derivative)	Zabeau and Stanley (1982)
N14-4	<i>uvrD thrA xyl7 ilvA metL arg1000 thi-i lysC</i> (W3623 derivative)	Ogawa et al. (1968)
YMC11	<i>Δ(glnG-glnA) thi endA hsr hutC_K Δlac U169</i>	Backman et al. (1981)

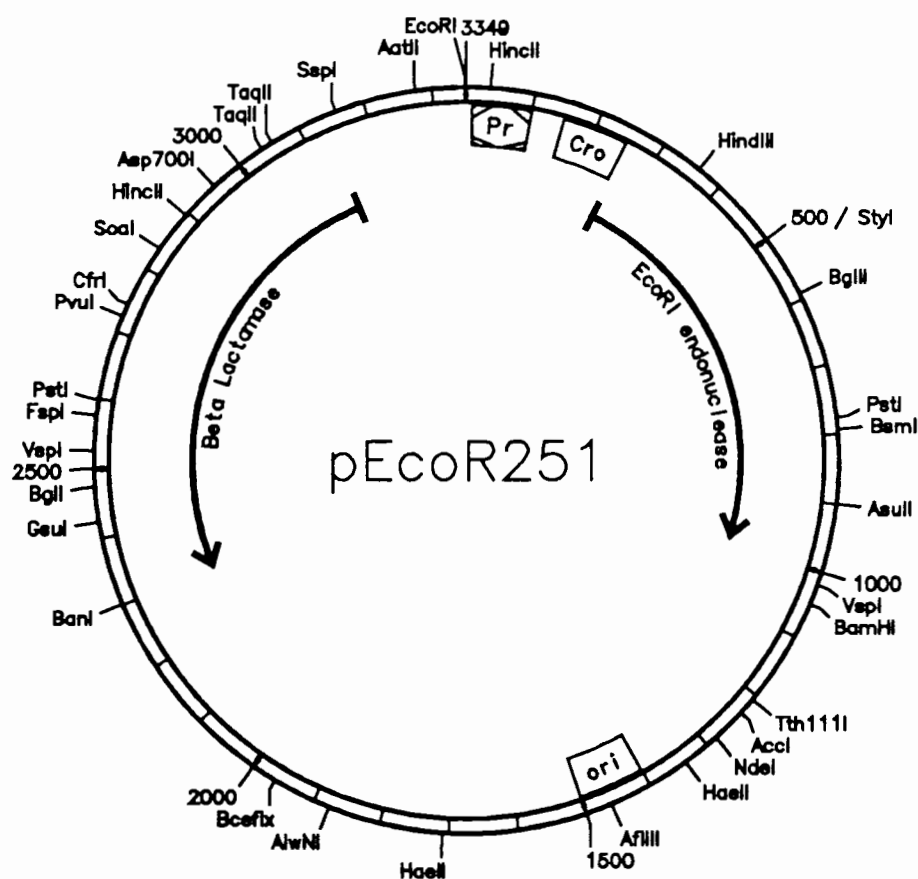
Appendix D

One- and three-letter codes used for amino acids

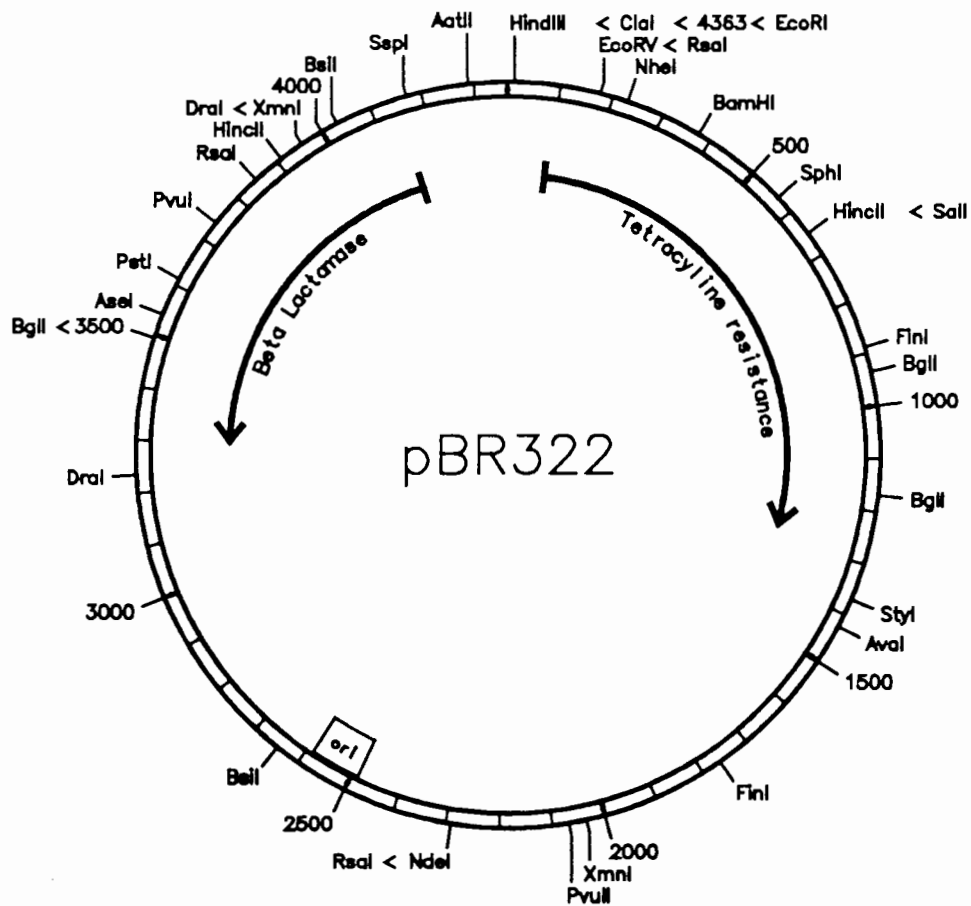
Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

Appendix E

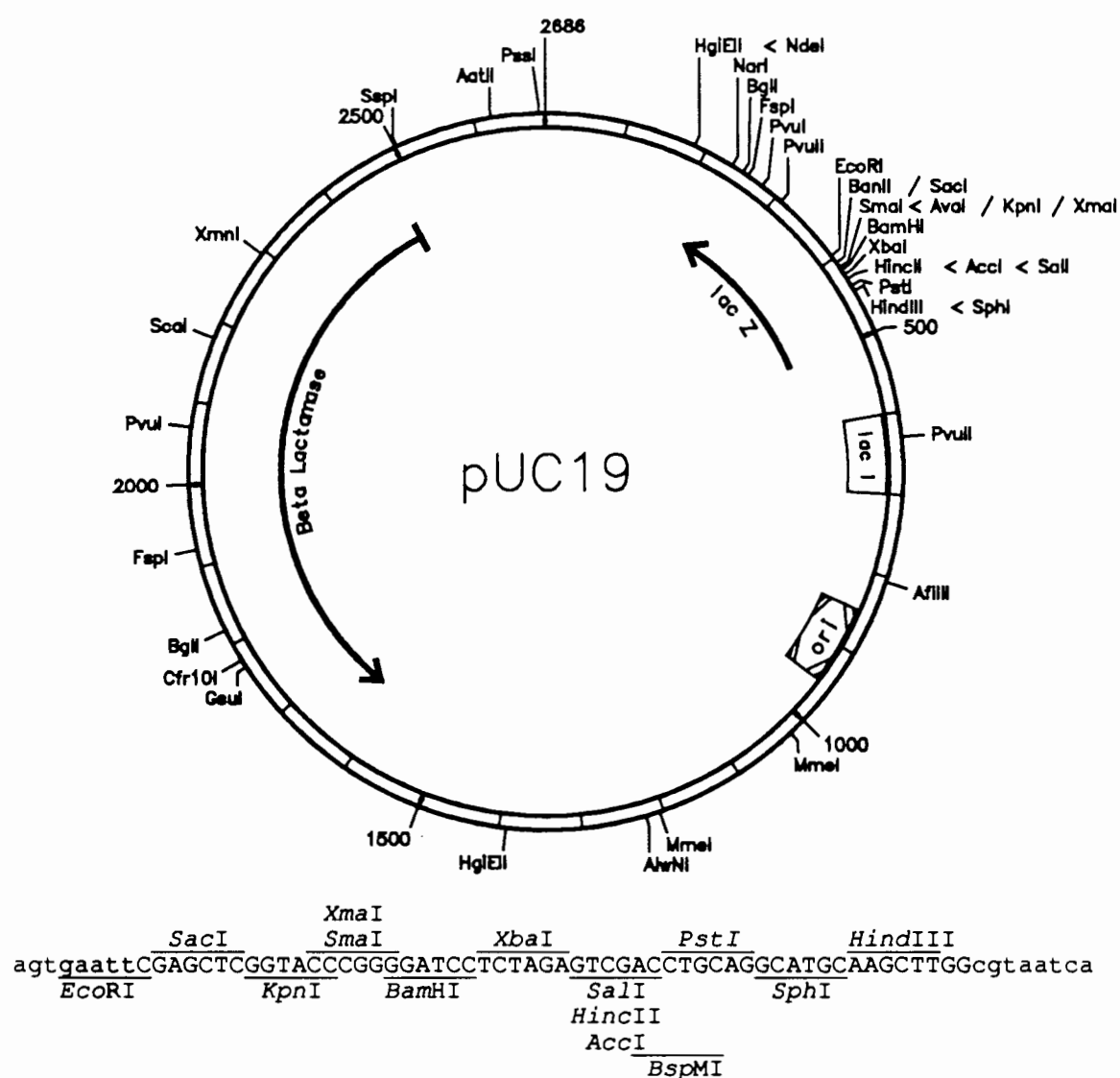
Plasmid vectors and phage restriction maps



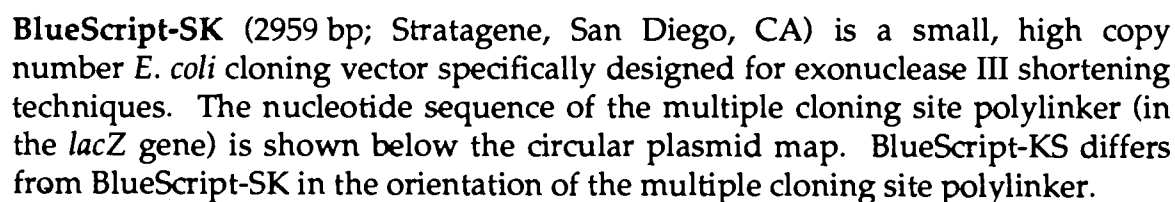
pEcoR251 (3349 bp) is a derivative of the pCL series vectors (Zabeau and Stanley, 1982), and was a gift from M. Zabeau, Biotechnology Business Development, Ghent, Belgium. This is a positive selection vector used routinely by insertional activation of the *EcoRI* endonuclease gene at either the *Bgl*III or *Hind*III site.

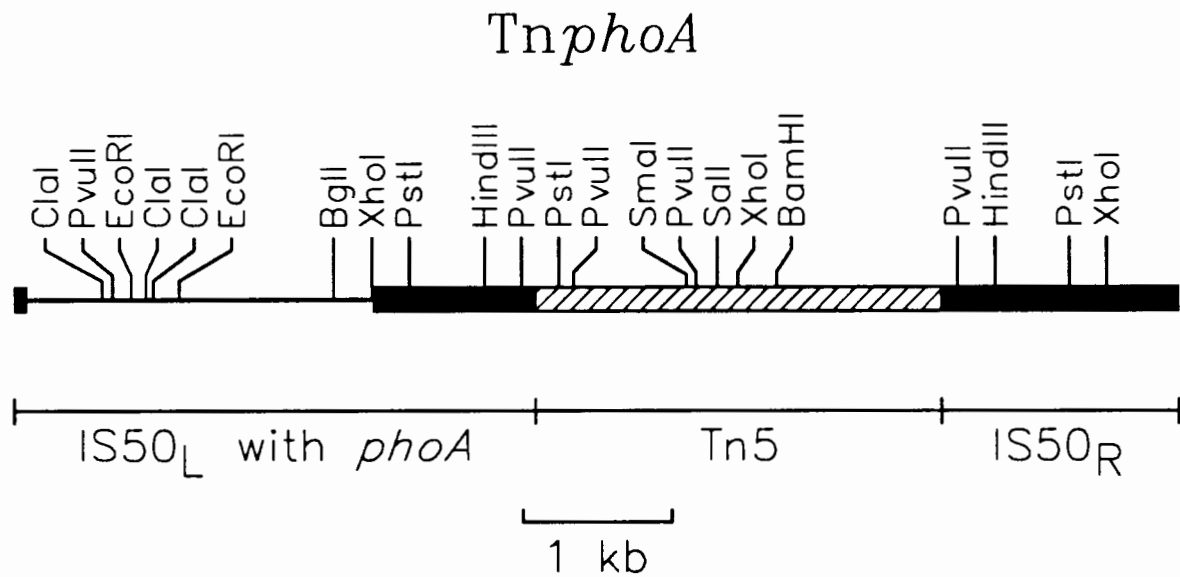


pBR322 (4361 bp; Sutcliffe, 1978) is a common *E. coli* cloning vector. This plasmid was constructed *in vitro* using the Tc^r gene from pSC101, the origin of DNA replication (ori) from the ColE1 derivative pMB1, and the Apr^r gene from transposon Tn3.



pUC19 (2686 bp; Yanisch-Perron et al., 1985) is a small, high copy number *E. coli* cloning vector. The pUC series of plasmids were constructed using portions of pBR322 and M13mp19. The nucleotide sequence of the multiple cloning site polylinker (in the *lacZ* gene) is shown below the circular plasmid map. pUC18 differs from pUC19 in the orientation of the multiple cloning site polylinker.





A partial restriction map of the transposon *Tn_{phoA}* (Manoil and Beckwith, 1985) showing the position of Tn5 relative to the leftward (IS50_L) and rightward (IS50_R) insertion sequences. IS50_L and IS50_R are represented as thick solid bars, Tn5 is represented as a thick slashed bar, and *phoA* is represented as a thin solid line.

Appendix F

Source code for chromatographic software

STARTUP.SRC

```

10  REM PROGRAM NAME = STARTUP.SRC
200 DEFINT A,C,H,L,P,V: DEFDBL D,K,R,S: DEFSTR E,F,I,Q,T,X,Y: DEFMSG B
210 CALL GHOME
220 PAGE=0: LINTS=1
230 CALL GPAGE(PAGE)
240 CALL LEVEL(LINTS)
250 CALL CLRSCL
260 CALL DISP(PAGE)
270 PAGE=1: LINTS=1
280 CALL GPAGE(PAGE)
290 CALL LEVEL(LINTS)
300 CALL CLRSCL
310 LX=135: LY=155
320 CALL MOVE(LX,LY)
330 LX=585: LY=155
340 CALL DLIN(LX,LY)
350 LX=585: LY=115
360 CALL DLIN(LX,LY)
370 LX=135: LY=115
380 CALL DLIN(LX,LY)
390 LX=135: LY=155
400 CALL DLIN(LX,LY)
410 LX=255: LY=155
420 CALL MOVE(LX,LY)
430 LX=255: LY=115
440 CALL DLIN(LX,LY)
450 LX=465: LY=155
460 CALL MOVE(LX,LY)
470 LX=465: LY=115
480 CALL DLIN(LX,LY)
490 LX=135: LY=150
500 CALL MOVE(LX,LY)
510 LX=120: LY=150
520 CALL DLIN(LX,LY)
530 LX=135: LY=120
540 CALL MOVE(LX,LY)
550 LX=120: LY=120
560 CALL DLIN(LX,LY)
570 LX=585: LY=150
580 CALL MOVE(LX,LY)
590 LX=600: LY=150
600 CALL DLIN(LX,LY)
610 LX=585: LY=120
620 CALL MOVE(LX,LY)
630 LX=600: LY=120
640 CALL DLIN(LX,LY)
650 LX=90: LY=155
660 CALL MOVE(LX,LY)
670 LX=120: LY=155
680 CALL DLIN(LX,LY)
690 LX=120: LY=115
700 CALL DLIN(LX,LY)
710 LX=90: LY=115
720 CALL DLIN(LX,LY)
730 LX=90: LY=155
740 CALL DLIN(LX,LY)
750 LX=600: LY=155
760 CALL MOVE(LX,LY)
770 LX=630: LY=155
780 CALL DLIN(LX,LY)
790 LX=630: LY=115
800 CALL DLIN(LX,LY)
810 LX=600: LY=115
820 CALL DLIN(LX,LY)
830 LX=600: LY=155
840 CALL DLIN(LX,LY)
850 LX=90: LY=145
860 CALL MOVE(LX,LY)
870 LX=60: LY=145
880 CALL DLIN(LX,LY)
890 LX=60: LY=120
900 CALL DLIN(LX,LY)
910 LX=80: LY=120
920 CALL DLIN(LX,LY)
930 LX=80: LY=150
940 CALL DLIN(LX,LY)
950 LX=60: LY=150
960 CALL DLIN(LX,LY)
970 LX=60: LY=125
980 CALL DLIN(LX,LY)
990 LX=90: LY=125
1000 CALL DLIN(LX,LY)
1010 LX=630: LY=145
1020 CALL MOVE(LX,LY)
1030 LX=660: LY=145
1040 CALL DLIN(LX,LY)
1050 LX=660: LY=120
1060 CALL DLIN(LX,LY)
1070 LX=640: LY=120
1080 CALL DLIN(LX,LY)
1090 LX=640: LY=150
1100 CALL DLIN(LX,LY)
1110 LX=660: LY=150
1120 CALL DLIN(LX,LY)
1130 LX=660: LY=125
1140 CALL DLIN(LX,LY)
1150 LX=630: LY=125
1160 CALL DLIN(LX,LY)
1163 PAGE=1
1166 CALL DISP(PAGE)
1170 LX=90: LY=114: LM=30: LB=15
1180 CALL BLKFIL(LX,LY,LM,LB)
1190 LX=120: LY=121: LM=15: LB=10
1200 CALL BLKFIL(LX,LY,LM,LB)
1210 LX=135: LY=114: LM=121: LB=15
1220 CALL BLKFIL(LX,LY,LM,LB)
1230 LX=465: LY=114: LM=120: LB=15
1240 CALL BLKFIL(LX,LY,LM,LB)
1250 LX=585: LY=121: LM=15: LB=10

```

```

1260 CALL BLKFIL(LX,LY,LM,LB)
1270 LX=600: LY=114: LM=31: LB=15
1280 CALL BLKFIL(LX,LY,LM,LB)
1290 LX=90: LY=170: LM=30: LB=15
1300 CALL BLKFIL(LX,LY,LM,LB)
1310 LX=120: LY=160: LM=15: LB=10
1320 CALL BLKFIL(LX,LY,LM,LB)
1330 LX=135: LY=170: LM=121: LB=15
1340 CALL BLKFIL(LX,LY,LM,LB)
1350 LX=465: LY=170: LM=120: LB=15
1360 CALL BLKFIL(LX,LY,LM,LB)
1370 LX=585: LY=160: LM=15: LB=10
1380 CALL BLKFIL(LX,LY,LM,LB)
1390 LX=600: LY=170: LM=31: LB=15
1400 CALL BLKFIL(LX,LY,LM,LB)
1470 LX=270: LY=50: EST="      "      HPLC      "
1480 CALL TEXTB(LX,LY,EST)
1490 LX=270: LY=64: EST="      "      SYSTEMS SOFTWARE      "
1500 CALL TEXTB(LX,LY,EST)
1550 LX=310: LY=210: EST="Version 1.2"
1560 CALL TEXTB(LX,LY,EST)
1570 LX=302: LY=250: EST="Press any key"
1580 CALL TEXTB(LX,LY,EST)
1590 IF INKEYS="" THEN 1590
1600 CHAIN "A:HPLC"

```

HPLC.SRC

```

10  REM PROGRAM NAME = HPLC.SRC
12  REM
15  REM *****
20  REM **
30  REM ** HPLC Systems software written for a Beckman 140M HPLC by **
40  REM ** Joseph D. Santangelo in the department of Microbiology at the **
50  REM ** University of Cape Town; Rondebosch 7700; South Africa; using **
60  REM ** an IBM-PC. **
70  REM **
80  REM ** Analog data from a Beckman 160 detector is converted to **
90  REM ** digital data by an Eagle Electronics PC-26 A/D converter. **
100 REM ** The base address of the PC-26 card must be selected as **
110 REM ** 700 (hex). **
120 REM **
130 REM ** These software programs run on IBM and compatible PC's, **
140 REM ** providing a Hercules monochrome graphics card is installed. **
150 REM **
160 REM **
170 REM **
180 REM *****
190 REM
200 DEFINT A,C,H,L,P,V: DEFDBL D,K,R,S: DEFSTR E,F,I,Q,T,X,Y: DEFMSG B
210 LINTS=1: PAGE=0
214 CALL GPAGE(PAGE)
216 CALL LEVEL(LINTS)
218 CALL CLRSCL
219 CALL DISP(PAGE)
220 REM ***** MENU GENERATION *****
230 LX=240: LY=24
231 CALL MOVE(LX,LY)
232 LX=478: LY=24
233 CALL DLIN(LX,LY)
234 LX=478: LY=44
235 CALL DLIN(LX,LY)
236 LX=240: LY=44
237 CALL DLIN(LX,LY)
238 LX=240: LY=22
239 CALL DLIN(LX,LY)
240 LX=478: LY=22
241 CALL DLIN(LX,LY)
242 LX=478: LY=46
243 CALL DLIN(LX,LY)
244 LX=240: LY=46
245 CALL DLIN(LX,LY)
247 LX=240: LY=44
248 CALL DLIN(LX,LY)
250 LX=240: LY=38: EST="HPLC DATA SYSTEMS MENU"
260 CALL TEXTB(LX,LY,EST)
270 LX=123: LY=87
271 CALL MOVE(LX,LY)
272 LX=596: LY=87
273 CALL DLIN(LX,LY)
274 LX=596: LY=289
275 CALL DLIN(LX,LY)
276 LX=123: LY=289
277 CALL DLIN(LX,LY)
278 LX=123: LY=85
279 CALL DLIN(LX,LY)
280 LX=596: LY=85
281 CALL DLIN(LX,LY)
282 LX=596: LY=291
283 CALL DLIN(LX,LY)
284 LX=123: LY=291
285 CALL DLIN(LX,LY)
286 LX=123: LY=289
287 CALL DLIN(LX,LY)
288 LX=158: LY=112: EST="1) Record Daily Baseline"
289 CALL TEXTB(LX,LY,EST)
290 LX=158: LY=139: EST="2) Record Calibration Chromatogram"
291 CALL TEXTB(LX,LY,EST)
292 LX=158: LY=166: EST="3) Data Collection and Manual Integration"
293 CALL TEXTB(LX,LY,EST)
294 LX=158: LY=193: EST="4) Data Collection with Automatic Integration"
295 CALL TEXTB(LX,LY,EST)
296 LX=158: LY=220: EST="5) Display a Stored Chromatogram"
297 CALL TEXTB(LX,LY,EST)
298 LX=158: LY=247: EST="6) Manually Integrate a Stored Chromatogram"
299 CALL TEXTB(LX,LY,EST)
300 LX=158: LY=274: EST="7) Exit to MS-DOS Operating System"
302 CALL TEXTB(LX,LY,EST)
310 LX=216: LY=329: EST="Enter Selection Number ( 1 - 7 )"
315 CALL TEXTB(LX,LY,EST)

```



```

320 LX=1: LY=343: EST=STRINGS(79,32)
330 CALL TEXTB(LX,LY,EST)
365 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
370 QS=INKEY$: IF QS="" THEN 370 ELSE CHOICE=VAL(QS)
380 LX=216: LY=329: EST=STRINGS(33,32)
385 CALL TEXTB(LX,LY,EST)
390 IF CHOICE<1 OR CHOICE>7 THEN GOSUB 600 ELSE 410
400 GOTO 310
410 ON CHOICE GOTO 460, 470, 480, 490, 500, 510, 520
430 REM
440 REM ***** GET SELECTED PROGRAM *****
450 REM
460 CHAIN "A:BASELINE"
470 CHAIN "A:CALIBRAT"
480 CHAIN "A:MANINT"
490 CHAIN "A:AUTOINT"
500 CHAIN "A:DISPLAY"
510 CHAIN "A:MANINTST"
520 REM
530 REM ***** EXIT FROM SPLC SYSTEMS *****
540 REM
550 LX=256: LY=343: EST="Confirm Selection (y/n)"
555 CALL TEXTB(LX,LY,EST)
560 QS=INKEY$: IF QS="" THEN 560
570 IF QS<>"Y" AND QS<>"y" THEN 310
575 CALL TMODE
577 CLOSE: CLS: END
580 REM
590 REM ***** ERROR SUBROUTINE *****
600 LX=292: LY=343: EST="INVALID ENTRY ": LINTS=0
602 CALL LEVEL(LINTS)
604 CALL TEXTB(LX,LY,EST)
606 LINTS=1
608 CALL LEVEL(LINTS)
610 BEEP: FOR LOOP = 1 TO 6000: NEXT
630 RETURN

MANINT.SRC

10 REM PROGRAM NAME = MANINT.SRC
94 REM
96 REM
100 REM ***** Initialize the 8255 *****
110 REM
120 OUT &B703,&B92
194 REM
196 REM
200 REM ***** Initilize the Screen and Dimension Variables *****
210 REM
220 DEFINT A,C,B,L,P,V: DEFDBL D,K,R,S: DEFSTR E,F,I,Q,T,X,Y: DEFUNG B
230 DIM ASTO(5010),BSTO(5010),PKSTART(1,25),PKEND(1,25),DPKAREA(25),DPKRT(25),
DPKAMT(25),EPKNAMS(25),STORT(20),STDXMEAN(20),STDYMEAN(20),STDSLOPE(20),
ENDNAMS(20)
240 PAGE=0: LINTS=1
250 CALL GPAGE(PAGE)
260 CALL LEVEL(LINTS)
270 CALL CLSCR
280 CALL DISP(PAGE)
284 REM
294 REM
300 REM ***** Convert Date Format from American to European *****
310 REM
320 EDATES = DATES: MIDS(EDATES,1,2) = MIDS(DATES,4,2): MIDS(EDATES,4,2) =
MIDS(DATES,1,2)
394 REM
396 REM
400 REM ***** Starting Prompts *****
410 REM
412 CALL CLSCR
413 LX=350: LY=30: EST="Data Collection and Manual Integration"
414 CALL TEXTB(LX,LY,EST)
415 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
416 FILES=""
418 LX=20: LY=180: EST="NAME OF FILE FOR DATA STORAGE (extension not
permitted) "
420 CALL TEXTB(LX,LY,EST)
421 QS=INKEY$: IF QS="" THEN 421
422 CHAN=ASC(QS)
423 IF (CHAN<13 AND CHAN<8) AND (CHAN<32 OR CHAN>126) THEN SOUND 40,10:
GOTO 421
424 IF CHAN=13 THEN 435
425 IF CHAN=8 AND LEN(FILES)<1 THEN SOUND 40,10: GOTO 421
426 IF CHAN=8 AND LEN(FILES)>0 THEN FILES=LEFT$(FILES,(LEN(FILES)-1)):
GOTO 428
427 FILES=FILES+QS
428 LX=335: LY=180: EST=FILES" "
429 CALL TEXTB(LX,LY,EST)
430 GOTO 421
435 IF MIDS(FILES,2,1) <> "." THEN FILES = "B:"+FILES
440 FOR LOOP = 1 TO LEN(FILES): IF MIDS(FILES,LOOP,1) <> "." THEN NEXT LOOP
442 ELSE 442
441 GOTO 450
442 LINTS=0
443 CALL LEVEL(LINTS)
444 LX=248: LY=328: EST="EXTENSION NOT PERMITTED "
445 CALL TEXTB(LX,LY,EST)
446 LINTS=1: BEEP
447 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
448 CALL LEVEL(LINTS)
449 GOTO 412
450 IF LEN(FILES) < 11 THEN 460
451 LINTS=0
452 CALL LEVEL(LINTS)
453 LX=256: LY=328: EST="FILE NAME IS TOO LONG "
454 CALL TEXTB(LX,LY,EST)
455 LINTS=1: BEEP
456 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
457 CALL LEVEL(LINTS)
458 GOTO 412
460 PAGE=1: LINTS=1
462 CALL GPAGE(PAGE)
464 CALL LEVEL(LINTS)
466 CALL CLSCR
468 LX=20: LY=180: EST="ARE YOU USING AN EXTERNAL TRIGGER TO START SAMPLING
(Y/N) ?"
470 CALL TEXTB(LX,LY,EST)
472 CALL DISP(PAGE)
480 EXTRS = INKEY$: IF EXTRS = "" THEN 480
490 IF EXTRS = "N" OR EXTRS = "n" OR EXTRS = "Y" OR EXTRS = "y" THEN 510
500 SOUND 40,10: PAGE=1
504 CALL CLSCR
506 GOTO 468
510 PAGE=0: LINTS=1
520 CALL GPAGE(PAGE)
530 CALL LEVEL(LINTS)
540 CALL CLSCR
594 REM
596 REM

600 REM ***** Draw Running Screen *****
610 REM
620 LX=30: LY=0: GOSUB 20010
630 LX=30: LY=260: GOSUB 20030
632 LX=630: LY=260: GOSUB 20030
634 FOR MARK = 256 TO 0 STEP -10
636 LX=30: LY=MARK: GOSUB 20010
638 LX=25: LY=MARK: GOSUB 20030
640 NEXT MARK
642 FOR MARK = 30 TO 630 STEP 20
644 LX=MARK: LY=260: GOSUB 20010
646 LX=MARK: LY=265: GOSUB 20030
648 NEXT MARK
650 FOR MARK = 30 TO 630 STEP 100
652 LX=MARK: LY=265: GOSUB 20010
654 LX=MARK: LY=270: GOSUB 20030
656 NEXT MARK
658 FOR MARK = 30 TO 630 STEP 200
660 LX=MARK: LY=270: GOSUB 20010
662 LX=MARK: LY=275: GOSUB 20030
664 NEXT MARK
666 LX=21: LY=291: EST="15": GOSUB 20050
668 LX=121: LY=291: EST="20": GOSUB 20050
670 LX=221: LY=291: EST="25": GOSUB 20050
672 LX=321: LY=291: EST="30": GOSUB 20050
674 LX=421: LY=291: EST="35": GOSUB 20050
676 LX=521: LY=291: EST="40": GOSUB 20050
678 LX=621: LY=291: EST="45": GOSUB 20050
680 LX=281: LY=305: EST="TIME (mins)": GOSUB 20050
690 LX=619: LY=17: EST="DATES: GOSUB 20050
700 LX=30: LY=343: EST="FILE = "FILES: GOSUB 20050
710 LX=450: LY=328: EST="Sample Number = ": GOSUB 20050
720 LX=450: LY=343: EST="Run Time = ": GOSUB 20050
730 CALL DISP(PAGE)
796 REM
800 REM ***** Sample Channel 0 -- External Trigger *****
810 REM
820 IF EXTRS = "Y" OR EXTRS = "y" THEN CHANNEL = 0 ELSE GOTO 900
830 VALUE=0
840 LX=30: LY=328: EST="Waiting for External Trigger.....": GOSUB 20050
850 WHILE VALUE < 2500
860 OUT &B702,2
863 OUT &B702,CHANNEL*810+3
866 VALUE=(INP(&B701)AND 4BF)*256+INP(&B700)
870 WEND
894 REM
896 REM
900 REM ***** Determine Injection Time of Chromatogram *****
910 REM
920 INJECTS = TIMES: MINS = INT(VAL(MIDS(INJECTS,4,2)))
930 IF MINS>24 THEN 1040 ELSE IF MINS>15 THEN 1010 ELSE IF MINS<15 THEN 970
940 REM ----- MINS = 15 -----
950 MIDS(INJECTS,4,2) = "00"
960 GOTO 1100
970 REM ----- MINS < 15 -----
980 IF VAL(MIDS(INJECTS,1,2)) > 10 THEN MIDS(INJECTS,1,2) =
MIDS(STR$(INT((VAL(MIDS(INJECTS,1,2))-1)),2),2) ELSE MIDS(INJECTS,1,2) =
"0"+MIDS(STR$(INT((VAL(MIDS(INJECTS,1,2))-1)),2),2)
990 MIDS(INJECTS,4,2) = MIDS(STR$(INT((VAL(MIDS(INJECTS,4,2))+45)),2),2)
1000 GOTO 1100
1010 REM ----- 15 < MINS < 24: single digit -----
1020 MIDS(INJECTS,4,2) = "0"+MIDS(STR$(INT((VAL(MIDS(INJECTS,4,2))-15)),2),1)
1030 GOTO 1100
1040 REM ----- MINS > 24 -----
1050 MIDS(INJECTS,4,2) = MIDS(STR$(INT((VAL(MIDS(INJECTS,4,2))-15)),2),2)
1094 REM
1096 REM
1100 REM ***** Sample Channel 1 -- Absorbance Data from SPLC *****
1110 REM
1120 CHANNEL=1: NOSAMPLES=0: LASTX=31: LASTY=256: RUNTIME=0: BSTO(0)=.006
1130 LX=30: LY=328: EST=STRINGS(35,32): GOSUB 20050
1140 LX=30: LY=328: EST="Sampling.....": GOSUB 20050
1150 STARTIME = TIMER
1155 LX=30: LY=256
1156 CALL MOVE(LX,LY)
1160 WHILE RUNTIME <= 45
1170 OUT &B702,2
1173 OUT &B702,CHANNEL*810+3
1176 VALUE=(INP(&B701)AND 4BF)*256+INP(&B700)
1180 NOSAMPLES = NOSAMPLES + 1
1190 ABVAL = 240 - ((CINT(VALUE/16))+4)
1200 KURRTIME = TIMER
1210 RUNTIME = ((KURRTIME-STARTIME)/60)+15
1215 IF RUNTIME < BSTO(NOSAMPLES-1)+.006 THEN 1200
1220 PLTIME = ((CINT((RUNTIME-15)*20))+30)
1230 LX=PLTIME: LY=ABVAL
1240 CALL DLIN(LX,LY)
1250 BSTO(NOSAMPLES) = RUNTIME: ASTO(NOSAMPLES) = ABVAL
1260 LX=540: LY=343: TEMPS=STR$(RUNTIME): EST=LEFT$(TEMPS,10): GOSUB 20050
1270 LX=585: LY=328: EST=STR$(NOSAMPLES): GOSUB 20050
1280 LX=628: LY=34: EST=TIMES: GOSUB 20050
1290 WEND
1294 REM
1296 REM
1300 REM ***** Store Data in a Sequential File (-FILES) *****
1310 REM
1320 LX=30: LY=328: EST=STRINGS(15,32): GOSUB 20050
1330 LX=381: LY=328: EST="Storing Data.....": GOSUB 20050
1335 FILEDATAB = FILES+".DAT"
1340 OPEN FILEDATAB FOR OUTPUT AS #1
1350 WRITE #1, EDATES, INJECTS, NOSAMPLES, KURRTIME, STARTIME
1360 FOR LOOP = 1 TO NOSAMPLES STEP 20
1370 B0 = BSTO(LOOP)
1371 A0 = ASTO(LOOP)
1372 B1 = BSTO(LOOP+1)
1373 A1 = ASTO(LOOP+1)
1374 B2 = BSTO(LOOP+2)
1375 A2 = ASTO(LOOP+2)
1376 B3 = BSTO(LOOP+3)
1377 A3 = ASTO(LOOP+3)
1378 B4 = BSTO(LOOP+4)
1379 A4 = ASTO(LOOP+4)
1380 B5 = BSTO(LOOP+5)
1381 A5 = ASTO(LOOP+5)
1382 B6 = BSTO(LOOP+6)
1383 A6 = ASTO(LOOP+6)
1384 B7 = BSTO(LOOP+7)
1385 A7 = ASTO(LOOP+7)
1386 B8 = BSTO(LOOP+8)
1387 A8 = ASTO(LOOP+8)
1388 B9 = BSTO(LOOP+9)
1389 A9 = ASTO(LOOP+9)
1390 B10 = BSTO(LOOP+10)
1391 A10 = ASTO(LOOP+10)
1392 B11 = BSTO(LOOP+11)
1393 A11 = ASTO(LOOP+11)
1394 B12 = BSTO(LOOP+12)
1395 A12 = ASTO(LOOP+12)
1396 B13 = BSTO(LOOP+13)
1397 A13 = ASTO(LOOP+13)
1398 B14 = BSTO(LOOP+14)

```

```

1399 A14 = ASTO(LOOP+14)
1400 B15 = BSTO(LOOP+15)
1401 A15 = ASTO(LOOP+15)
1402 B16 = BSTO(LOOP+16)
1403 A16 = ASTO(LOOP+16)
1404 B17 = BSTO(LOOP+17)
1405 A17 = ASTO(LOOP+17)
1406 B18 = BSTO(LOOP+18)
1407 A18 = ASTO(LOOP+18)
1408 B19 = BSTO(LOOP+19)
1409 A19 = ASTO(LOOP+19)
1410 WRITE #1,B0,A0,B1,A1,B2,A2,B3,A3,B4,A4,B5,A5,B6,A6,B7,A7,B8,A8,B9,
      A9,B10,A10,B11,A11,B12,A12,B13,A13,B14,A14,B15,A15,B16,A16,B17,A17,
      B18,A18,B19,A19
1420 NEXT LOOP
1430 CLOSE
1600 LX=30: LY=328: EST=STRINGS(20,32): GOSUB 20050
1605 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
1610 LX=18: LY=328: EST="Raw data is stored, do you wish to continue ...
      Manual Integration (y/n) ?": GOSUB 20050
1615 BEEP
1620 QS=INKEYS: IF QS="" THEN 1620
1630 IF QS = "Y" OR QS = "y" THEN 1700
1640 IF QS = "N" OR QS = "n" THEN 5020
1650 SOUND 40,10: GOTO 1620
1694 REM
1696 REM
1700 REM ##### Manual Integration #####
1710 REM
1720 LX=18: LY=328: EST=STRINGS(74,32): GOSUB 20050
1730 LX=10: LY=0
1740 CALL MOVE(LX,LY)
1750 LX=10: LY=259
1760 CALL DLINE(LX,LY)
1770 LINTS=2
1780 CALL LEVEL(LINTS)
1890 POSX=10: NUMSTRS=0: NUMENDS=0
1894 PLTLEN = CINT((BSTO(10)-15)*20)+30)
1896 PLTEND = CINT((BSTO(NOSAMPLS-10)-15)*20)+30)
1900 SAMPPOINT = CDBL((NOSAMPLS-20)/(PLTEND-PLTLEN))
1910 GOSUB 10130
1930 REM ----- Input Control Command -----
1940 IMOVES = INKEYS: IF IMOVES = "" THEN 1940
1942 IF LEN(IMOVES)>1 AND IMOVES=CHR$(0)+"M" THEN MOVE=28: GOTO 1980
1943 IF LEN(IMOVES)>1 AND IMOVES=CHR$(0)+"K" THEN MOVE=29: GOTO 1980
1945 MOVE=ASC(IMOVES)
1950 IF MOVE < 52 THEN 1940
1960 IF MOVE < 55 THEN 1980
1970 IF MOVE=65 AND POSX=29 AND POSX<631 THEN 2070 ELSE SOUND 40,10:
      GOTO 1940
1980 REM ----- Move Cursor -----
1990 IF MOVE = 28 THEN POSX2=POSX+1: GOTO 2000
1992 IF MOVE = 54 THEN POSX2=POSX+10: GOTO 2000
1994 IF MOVE = 29 THEN POSX2=POSX-1: GOTO 2000
1996 IF MOVE = 52 THEN POSX2=POSX-10: GOTO 2000
1998 SOUND 40,10: GOTO 1940
2000 IF POSX2<10 THEN BEEP: POSX2=10
2002 IF POSX2>640 THEN BEEP: POSX2=640
2004 LX=POSX: LY=0
2006 CALL MOVE(LX,LY)
2008 LX=POSX: LY=259
2010 CALL DLINE(LX,LY)
2012 LX=POSX2: LY=259
2014 CALL MOVE(LX,LY)
2016 LX=POSX2: LY=0
2018 CALL DLINE(LX,LY)
2020 POSX=POSX2
2030 GOTO 1940
2040 REM ----- Integration Control Commands -----
2050 REM
2060 REM ----- Peak Start -----
2070 IF MOVE = 82 OR MOVE = 115 THEN SOUND 523.25,.5 ELSE 2230
2080 LOOP = CINT((POSX-15)*SAMPPOINT)
2090 IF LOOP < 1 THEN LOOP = 1
2100 IF LOOP > NOSAMPLS THEN LOOP = NOSAMPLS
2110 LOWPOINT=0: FLAGS="FIRST"
2120 COMFX = CINT((BSTO(LOOP)-15)*20)+30)
2130 WHILE COMFX <= POSX
2140 IF COMFX < POSX THEN 2180
2150 IF FLAGS="FIRST" THEN NUMSTRS=NUMSTRS+1: PKSTART(0,NUMSTRS)=LOOP:
      FLAGS="NO"
2160 PKSTART(1,NUMSTRS)=LOOP
2170 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2180 LOOP = LOOP + 1
2190 COMFX = CINT((BSTO(LOOP)-15)*20)+30)
2200 WEND
2210 LX=POSX: LY=LOWPOINT+1
2211 CALL MOVE(LX,LY)
2212 LX=POSX: LY=LOWPOINT+11
2213 CALL DLINE(LX,LY)
2214 LX=POSX+4: LY=LOWPOINT+11
2215 CALL DLINE(LX,LY)
2216 GOTO 1940
2220 REM ----- Peak End -----
2230 IF MOVE = 69 OR MOVE = 101 THEN SOUND 523.25,.5 ELSE 2390
2240 LOOP = CINT((POSX-25)*SAMPPOINT)
2250 IF LOOP > NOSAMPLS THEN LOOP = NOSAMPLS
2260 IF LOOP < 1 THEN LOOP = 1
2270 LOWPOINT=0: FLAGS="FIRST"
2280 COMFX = CINT((BSTO(LOOP)-15)*20)+30)
2290 WHILE COMFX >= POSX
2300 IF COMFX > POSX THEN 2340
2310 IF FLAGS="FIRST" THEN NUMENDS=NUMENDS+1: PKEND(0,NUMENDS)=LOOP:
      FLAGS="NO"
2320 PKEND(1,NUMENDS)=LOOP
2330 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2340 LOOP = LOOP - 1
2350 COMFX = CINT((BSTO(LOOP)-15)*20)+30)
2360 WEND
2370 LX=POSX: LY=LOWPOINT+1
2371 CALL MOVE(LX,LY)
2372 LX=POSX: LY=LOWPOINT+11
2373 CALL DLINE(LX,LY)
2374 LX=POSX+4: LY=LOWPOINT+11
2375 CALL DLINE(LX,LY)
2376 GOTO 1940
2380 REM ----- Clear Entry -----
2390 IF MOVE < 67 AND MOVE < 99 THEN 2740
2400 CANCEL=0: LOWPOINT=0
2410 IF NUMSTRS < 1 THEN 2570
2420 FOR LOOP = 1 TO NUMSTRS
2430 COMFX = CINT((BSTO(PKSTART(0,LOOP))-15)*20)+30)
2440 IF COMFX = POSX THEN CANCEL = LOOP
2450 NEXT LOOP
2460 IF CANCEL < 1 THEN 2570
2470 FOR LOOP = PKSTART(0,CANCEL) TO PKSTART(1,CANCEL)
2480 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2490 NEXT LOOP
2500 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2510 LX=POSX: LY=LOWPOINT+1
2511 CALL MOVE(LX,LY)
2512 LX=POSX: LY=LOWPOINT+11
2513 CALL DLINE(LX,LY)
2514 LX=POSX+4: LY=LOWPOINT+11
2515 CALL DLINE(LX,LY)
2520 FOR LOOP = CANCEL TO NUMSTRS-1
2530 PKSTART(0,LOOP) = PKSTART(0,LOOP+1)
2540 PKSTART(1,LOOP) = PKSTART(1,LOOP+1)
2550 NEXT LOOP
2560 NUMSTRS = NUMSTRS - 1: GOTO 1940
2570 IF NUMSTRS < 1 AND NUMENDS<1 THEN SOUND 40,10: GOTO 1940
2580 FOR LOOP = 1 TO NUMENDS
2590 COMFX = CINT((BSTO(PKEND(0,LOOP))-15)*20)+30)
2600 IF COMFX = POSX THEN CANCEL = LOOP
2610 NEXT LOOP
2620 IF CANCEL < 1 THEN SOUND 40,10: GOTO 1940
2630 FOR LOOP = PKEND(1,CANCEL) TO PKEND(0,CANCEL)
2640 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2650 NEXT LOOP
2660 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2670 LX=POSX: LY=LOWPOINT+1
2671 CALL MOVE(LX,LY)
2672 LX=POSX: LY=LOWPOINT+11
2673 CALL DLINE(LX,LY)
2674 LX=POSX+4: LY=LOWPOINT+11
2675 CALL DLINE(LX,LY)
2680 FOR LOOP = CANCEL TO NUMENDS-1
2690 PKEND(0,LOOP) = PKEND(0,LOOP+1)
2700 PKEND(1,LOOP) = PKEND(1,LOOP+1)
2710 NEXT LOOP
2720 NUMENDS = NUMENDS - 1: GOTO 1940
2730 REM ----- Redo From Start -----
2740 IF MOVE <> 82 AND MOVE <> 114 THEN 2840
2750 BEEP: LINTS=1
2751 CALL LEVEL(LINTS)
2752 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2753 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2754 LX=30: LY=328: EST="Redo From Start.....": GOSUB 20050
2755 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2760 QS = INKEYS: IF QS = "" THEN 2760
2770 IF QS = "Y" OR QS = "y" THEN 2790
2780 GOSUB 10130: GOTO 1940
2790 CALL CLRSCL
2791 LX=30: LY=0: GOSUB 20010
2792 LX=30: LY=260: GOSUB 20030
2793 LX=630: LY=260: GOSUB 20030
2794 FOR MARK = 256 TO 0 STEP -10
2795 LX=30: LY=MARK: GOSUB 20010
2796 LX=25: LY=MARK: GOSUB 20030
2797 NEXT MARK
2798 FOR MARK = 30 TO 630 STEP 20
2799 LX=MARK: LY=260: GOSUB 20010
2800 LX=MARK: LY=265: GOSUB 20030
2801 NEXT MARK
2802 FOR MARK = 30 TO 630 STEP 100
2803 LX=MARK: LY=265: GOSUB 20010
2804 LX=MARK: LY=270: GOSUB 20030
2805 NEXT MARK
2806 FOR MARK = 30 TO 630 STEP 200
2807 LX=MARK: LY=270: GOSUB 20010
2808 LX=MARK: LY=275: GOSUB 20030
2809 NEXT MARK
2810 LX=21: LY=291: EST="15": GOSUB 20050
2811 LX=121: LY=291: EST="20": GOSUB 20050
2812 LX=221: LY=291: EST="25": GOSUB 20050
2813 LX=321: LY=291: EST="30": GOSUB 20050
2814 LX=421: LY=291: EST="35": GOSUB 20050
2815 LX=521: LY=291: EST="40": GOSUB 20050
2816 LX=621: LY=291: EST="45": GOSUB 20050
2817 LX=281: LY=305: EST="TIME (mins)": GOSUB 20050
2818 LX=(CINT((BSTO(1)-15)*20)+30): LY=ASTO(1)
2819 CALL PLOT(LX,LY)
2820 CALL MOVE(LX,LY)
2821 FOR LOOP = 2 TO NOSAMPLS
2822 LX=(CINT((BSTO(LOOP)-15)*20)+30): LY=ASTO(LOOP)
2823 CALL DLINE(LX,LY)
2824 NEXT LOOP
2825 GOTO 1700
2830 REM ----- Quit Integration -----
2840 IF MOVE <> 81 AND MOVE <> 113 THEN 2900
2850 BEEP: LINTS=1
2851 CALL LEVEL(LINTS)
2852 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2853 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2854 LX=30: LY=328: EST="Quit.....": GOSUB 20050
2855 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2860 QS = INKEYS: IF QS = "" THEN 2860
2870 IF QS<>"Y" AND QS<>"y" THEN 2880
2872 CALL CLRSCL
2874 CALL TMOOE
2876 RUN "A:BPIC"
2880 GOSUB 10130: GOTO 1940
2890 REM ----- Process Data -----
2900 IF MOVE <> 80 AND MOVE <> 112 THEN SOUND 40,10: GOTO 1940
2910 BEEP: LINTS=1
2911 CALL LEVEL(LINTS)
2912 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2913 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2914 LX=30: LY=328: EST="Process.....": GOSUB 20050
2915 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2920 QS = INKEYS: IF QS = "" THEN 2920
2930 IF QS = "Y" OR QS = "y" THEN 2950
2940 GOSUB 10130: GOTO 1940
2950 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2955 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2960 IF NUMSTRS = NUMENDS THEN 3020
2970 BEEP: IF NUMSTRS>NUMENDS THEN 2980
2975 LX=137: LY=328: EST="NUMBER OF PEAK STARTS < NUMBER OF PEAK ENDS":
      GOSUB 20050
2977 GOTO 2990
2980 LX=137: LY=328: EST="NUMBER OF PEAK STARTS > NUMBER OF PEAK ENDS":
      GOSUB 20050
2990 LX=95: LY=343: EST="Press any key to continue.....": GOSUB 20050
3000 IF INKEYS="" THEN 3000
3010 GOSUB 10130: GOTO 1940
3020 REM ----- Bubble Sort -----
3030 REM
3040 CHECK = 1
3050 WHILE CHECK
3060 CHECK = 0
3070 FOR LOOP = 1 TO NUMSTRS-1
3080 IF BSTO(PKSTART(0,LOOP)) <= BSTO(PKSTART(0,LOOP+1)) THEN 3120
3090 SWAP PKSTART(0,LOOP),PKSTART(0,LOOP+1)
3100 SWAP PKSTART(1,LOOP),PKSTART(1,LOOP+1)
3110 CHECK = 1
3120 NEXT LOOP
3130 WEND
3140 CHECK = 1
3150 WHILE CHECK
3160 CHECK = 0
3170 FOR LOOP = 1 TO NUMENDS-1
3180 IF BSTO(PKEND(0,LOOP)) <= BSTO(PKEND(0,LOOP+1)) THEN 3220

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3190 SWAP PKEND(0,LOOP),PKEND(0,LOOP+1)
3200 SWAP PKEND(1,LOOP),PKEND(1,LOOP+1)
3210 CHECK = 1
3220 NEXT LOOP
3230 WEND
3240 REM ----- Confirm Order -----
3250 FOR LOOP = 1 TO NUMSTRTS
3260 IF PKSTART(0,LOOP) <= PKEND(0,LOOP) THEN 3280
3270 CHECK = CHECK + 1
3280 NEXT LOOP
3290 IF CHECK < 1 THEN 3360
3300 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3310 BEEP: LX=87: LY=328: EST="PEAK STARTS AND PEAK ENDS ARE NOT IN THE
CORRECT ORDER": GOSUB 20050
3320 LX=95: LY=343: EST="Press any key to continue.....": GOSUB 20050
3330 IF INKEY$="" THEN 3330
3340 GOSUB 10130: GOTO 1940
3350 REM ----- Integration -----
3360 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3364 LX=145: LY=328: EST="Calculating area for peak number": GOSUB 20050
3366 LX=473: LY=328: EST="of+STRS(NUMSTRTS): GOSUB 20050
3380 LINTS=2
3381 CALL LEVEL(LINTS)
3382 LX=POSX: LY=0
3383 CALL MOVE(LX,LY)
3384 LX=POSX: LY=259
3385 CALL DLINE(LX,LY)
3386 LINTS=1
3387 CALL LEVEL(LINTS)
3390 FOR LOOP1 = 1 TO NUMSTRTS
3400 LX=435: LY=328: EST=STRS(LOOP1)
3405 CALL TEXTS(LX,LY,EST)
3410 REM ----- Baseline (LSRL) Calculations -----
3420 DSUMX=0: DSUMY=0: DXY=0: DXSQ=0: SIGRPOINT=675: FLAGS="NO"
3430 DXMEAN=0: DYMEAN=0: DLXY=0: DLXSQ=0: LOWPOINT=0
3440 FOR LOOP2 = PKSTART(0,LOOP1) TO PKSTART(1,LOOP1)
3450 DSUMX = DSUMX + BSTO(LOOP2)
3460 DSUMY = DSUMY + ASTO(LOOP2)
3470 DXY = DXY + BSTO(LOOP2)*ASTO(LOOP2)
3480 DXSQ = DXSQ + BSTO(LOOP2)^2
3490 IF ASTO(LOOP2) > LOWPOINT THEN LOWPOINT = ASTO(LOOP2)
3500 NEXT LOOP2
3510 PTSTRT = ((CINT((BSTO(PKSTART(0,LOOP1))-15)*20)+30)
3520 LINTS=2
3522 CALL LEVEL(LINTS)
3523 LX=PTSTRT: LY=LOWPOINT+1
3524 CALL MOVE(LX,LY)
3525 LX=PTSTRT: LY=LOWPOINT+11
3526 CALL DLINE(LX,LY)
3527 LX=PTSTRT+4: LY=LOWPOINT+11
3528 CALL DLINE(LX,LY)
3530 LOWPOINT = 0
3540 FOR LOOP3 = PKEND(1,LOOP1) TO PKEND(0,LOOP1)
3550 DSUMX = DSUMX + BSTO(LOOP3)
3560 DSUMY = DSUMY + ASTO(LOOP3)
3570 DXY = DXY + BSTO(LOOP3)*ASTO(LOOP3)
3580 DXSQ = DXSQ + BSTO(LOOP3)^2
3590 IF ASTO(LOOP3) > LOWPOINT THEN LOWPOINT = ASTO(LOOP3)
3600 NEXT LOOP3
3610 PTEND = ((CINT((BSTO(PKEND(0,LOOP1))-15)*20)+30)
3620 LX=PTEND: LY=LOWPOINT+1
3622 CALL MOVE(LX,LY)
3623 LX=PTEND: LY=LOWPOINT+11
3624 CALL DLINE(LX,LY)
3625 LX=PTEND+4: LY=LOWPOINT+11
3626 CALL DLINE(LX,LY)
3627 LINTS=1
3628 CALL LEVEL(LINTS)
3630 W=PKSTART(1,LOOP1)-PKSTART(0,LOOP1)+PKEND(0,LOOP1)-PKEND(1,LOOP1)+2
3640 DXMEAN = DSUMX/W: DYMEAN = DSUMY/W
3650 DLXY=DKY-(DSUMX*DSUMY/W): DLXSQ=DXSQ-((DSUMX^2)/W): SLOPE=DLXY/DLXSQ
3660 REM ----- Calculate Area & Retention Time -----
3670 FOR LOOP4 = PKSTART(0,LOOP1) TO PKEND(0,LOOP1)-1
3690 DBASL = DYMEAN+SLOPE*(BSTO(LOOP4)-DXMEAN)
3700 DBASR = DYMEAN+SLOPE*(BSTO(LOOP4+1)-DXMEAN)
3710 DUPRSD = ABS(ASSTO(LOOP4)-ASTO(LOOP4+1))
3720 DBASID = ABS(DBASL-DBASR)
3730 IF ASTO(LOOP4)<ASTO(LOOP4+1) THEN DTEMPY1=ASTO(LOOP4+1) ELSE
DTEMPY1=ASTO(LOOP4)
3740 IF DBASL>DBASR THEN DTEMPY2=DBASR ELSE DTEMPY2=DBASL
3750 DPKAREA(LOOP1)=DPKAREA(LOOP1)+((BSTO(LOOP4+1)-BSTO(LOOP4))
*((.5*DUPRSD)+(1.5*DBASID)*DTEMPY2-DTEMPY1))
3760 IF ASTO(LOOP4) >= HIGHPOINT THEN 3780
3770 DSTATRT=BSTO(LOOP4): HIGHPOINT=ASTO(LOOP4): FLAGS="END"
3780 IF ASTO(LOOP4) >= HIGHPOINT OR FLAGS<>"END" THEN 3800
3790 DENORT=BSTO(LOOP4-1): FLAGS="NO"
3800 NEXT LOOP4
3810 DPKRT(LOOP1) = DSTATRT+((DENORT-DSTATRT)/2)
3820 REM ----- Print Retention Times on Screen -----
3830 IS = STRS(DPKRT(LOOP1))
3840 PTX = CINT((DPKRT(LOOP1)-15)*20)+27
3850 IF HIGHPOINT < 27 THEN PTY = 27 ELSE PTY = HIGHPOINT
3860 FOR LOOP5 = 2 TO 6
3870 IF MID$(IS,LOOP5,1) = "." THEN PTY=PTY-3: GOTO 3920
3880 CHARACTER = VAL(MID$(IS,LOOP5,1))+1
3890 PTY = PTY + 6
3900
3910 ON CHARACTER GOTO 3923,3932,3938,3947,3960,3966,3977,3988,
3994,4009
3920 LX=PTX+6: LY=PTY: GOSUB 20010
3921 LX=PTX+5: LY=PTY: GOSUB 20030
3922 GOTO 4030
3923 LX=PTX+1: LY=PTY: GOSUB 20010
3924 LX=PTX+3: LY=PTY: GOSUB 20030
3925 LX=PTX+6: LY=PTY+1: GOSUB 20030
3926 LX=PTX+6: LY=PTY+2: GOSUB 20030
3927 LX=PTX+5: LY=PTY+3: GOSUB 20030
3928 LX=PTX+1: LY=PTY+3: GOSUB 20030
3929 LX=PTX: LY=PTY+2: GOSUB 20030
3930 LX=PTX: LY=PTY+1: GOSUB 20030
3931 GOTO 4030
3932 LX=PTX+6: LY=PTY+3: GOSUB 20010
3933 LX=PTX+6: LY=PTY: GOSUB 20030
3934 LX=PTX+5: LY=PTY+1: GOSUB 20030
3935 LX=PTX: LY=PTY+1: GOSUB 20030
3936 LX=PTX+2: LY=PTY+3: GOSUB 20030
3937 GOTO 4030
3938 LX=PTX+6: LY=PTY: GOSUB 20010
3939 LX=PTX+6: LY=PTY+3: GOSUB 20030
3940 LX=PTX+5: LY=PTY+3: GOSUB 20030
3941 LX=PTX+2: LY=PTY: GOSUB 20030
3942 LX=PTX+1: LY=PTY: GOSUB 20030
3943 LX=PTX: LY=PTY+1: GOSUB 20030
3944 LX=PTX: LY=PTY+2: GOSUB 20030
3945 LX=PTX+1: LY=PTY+3: GOSUB 20030
3946 GOTO 4030
3947 LX=PTX+1: LY=PTY+3: GOSUB 20010
3948 LX=PTX: LY=PTY+2: GOSUB 20030
3949 LX=PTX: LY=PTY+1: GOSUB 20030
3950 LX=PTX+1: LY=PTY: GOSUB 20030
3951 LX=PTX+2: LY=PTY: GOSUB 20030
3952 LX=PTX+3: LY=PTY+1: GOSUB 20030
3953 LX=PTX+3: LY=PTY+2: GOSUB 20030
3954 LX=PTX+4: LY=PTY: GOSUB 20010
3955 LX=PTX+5: LY=PTY: GOSUB 20030
3956 LX=PTX+6: LY=PTY+1: GOSUB 20030
3957 LX=PTX+6: LY=PTY+2: GOSUB 20030
3958 LX=PTX+5: LY=PTY+3: GOSUB 20030
3959 GOTO 4030
3960 LX=PTX+1: LY=PTY+3: GOSUB 20010
3961 LX=PTX+4: LY=PTY+3: GOSUB 20030
3962 LX=PTX+4: LY=PTY: GOSUB 20030
3963 LX=PTX+6: LY=PTY+1: GOSUB 20010
3964 LX=PTX: LY=PTY+1: GOSUB 20030
3965 GOTO 4030
3966 LX=PTX: LY=PTY: GOSUB 20010
3967 LX=PTX: LY=PTY+3: GOSUB 20030
3968 LX=PTX+2: LY=PTY+3: GOSUB 20030
3969 LX=PTX+2: LY=PTY+1: GOSUB 20030
3970 LX=PTX+3: LY=PTY: GOSUB 20030
3971 LX=PTX+5: LY=PTY: GOSUB 20030
3972 LX=PTX+6: LY=PTY+1: GOSUB 20030
3973 LX=PTX+6: LY=PTY+2: GOSUB 20030
3974 LX=PTX+5: LY=PTY+3: GOSUB 20030
3975 LX=PTX+4: LY=PTY+3: GOSUB 20030
3976 GOTO 4030
3977 LX=PTX+1: LY=PTY: GOSUB 20010
3978 LX=PTX: LY=PTY+1: GOSUB 20030
3979 LX=PTX+2: LY=PTY+3: GOSUB 20030
3980 LX=PTX+5: LY=PTY+3: GOSUB 20030
3981 LX=PTX+6: LY=PTY+2: GOSUB 20030
3982 LX=PTX+6: LY=PTY+1: GOSUB 20030
3983 LX=PTX+5: LY=PTY: GOSUB 20030
3984 LX=PTX+4: LY=PTY: GOSUB 20030
3985 LX=PTX+3: LY=PTY+1: GOSUB 20030
3986 LX=PTX+3: LY=PTY+2: GOSUB 20030
3987 GOTO 4030
3988 LX=PTX: LY=PTY+3: GOSUB 20010
3989 LX=PTX: LY=PTY: GOSUB 20030
3990 LX=PTX+2: LY=PTY: GOSUB 20030
3991 LX=PTX+5: LY=PTY+3: GOSUB 20030
3992 LX=PTX+6: LY=PTY+3: GOSUB 20030
3993 GOTO 4030
3994 LX=PTX+2: LY=PTY+3: GOSUB 20010
3995 LX=PTX+1: LY=PTY+3: GOSUB 20030
3996 LX=PTX: LY=PTY+2: GOSUB 20030
3997 LX=PTX: LY=PTY+1: GOSUB 20030
3998 LX=PTX+1: LY=PTY: GOSUB 20030
3999 LX=PTX+2: LY=PTY: GOSUB 20030
4000 LX=PTX+3: LY=PTY+1: GOSUB 20030
4001 LX=PTX+3: LY=PTY+2: GOSUB 20030
4002 LX=PTX+4: LY=PTY+3: GOSUB 20030
4003 LX=PTX+5: LY=PTY+3: GOSUB 20030
4004 LX=PTX+6: LY=PTY+2: GOSUB 20030
4005 LX=PTX+6: LY=PTY+1: GOSUB 20030
4006 LX=PTX+5: LY=PTY: GOSUB 20030
4007 LX=PTX+4: LY=PTY: GOSUB 20030
4008 GOTO 4030
4009 LX=PTX+6: LY=PTY+2: GOSUB 20010
4010 LX=PTX+4: LY=PTY: GOSUB 20030
4011 LX=PTX+1: LY=PTY: GOSUB 20030
4012 LX=PTX: LY=PTY+1: GOSUB 20030
4013 LX=PTX: LY=PTY+2: GOSUB 20030
4014 LX=PTX+1: LY=PTY+3: GOSUB 20030
4015 LX=PTX+2: LY=PTY+3: GOSUB 20030
4016 LX=PTX+3: LY=PTY+2: GOSUB 20030
4017 LX=PTX+3: LY=PTY+1: GOSUB 20030
4030 NEXT LOOP5
4040 NEXT LOOP1
4100 REM ----- Print Hardcopy of Chromatogram -----
4110 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4112 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4114 LX=619: LY=17: EST=STRINGS(10,32): GOSUB 20050
4116 LX=628: LY=34: EST=STRINGS(8,32): GOSUB 20050
4118 BEEP: LX=96: LY=328: EST="Press <shift> Frc5c-1> when this message
disappears": GOSUB 20050
4120 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4130 IF INKEY$="" THEN 4130
4140 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4150 FOR LOOP = 1 TO 5000: NEXT LOOP
4160 BEEP: LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4170 IF INKEY$="" THEN 4170
4180 REM ----- Quantitation -----
4190 REM
4200 REM ----- Read Standard Curve Parameters from A:STANDARDS.DAT -----
4210 LX=30: LY=328: EST="Loading Standard Curve Data, Please Wait.....":
GOSUB 20050
4230 OPEN "A:STANDARDS.DAT" AS #2 LEN = 35
4240 FIELD #2, 2 AS ENUMSTDS, 10 AS EDATIS, 23 AS EDUMHYS
4250 GET #2,1
4260 NUMSTDS = CVAL(ENUMSTDS): ESTDATES = EDATIS
4280 FIELD #2, 8 AS ERTS, 8 AS XMEANS, 8 AS YMEANS, 8 AS ESLOPES, 3 AS ENAMES
4290 FOR LOOP = 1 TO NUMSTDS
4300 GET #2,LOOP+1
4310 STDT(LOOP) = CVAL(ERTS)
4320 STDYMEAN(LOOP) = CVAL(XMEANS)
4330 STDYMEAN(LOOP) = CVAL(YMEANS)
4340 STDOSLOPE(LOOP) = CVAL(ESLOPES)
4350 ESTOMHYS(LOOP) = ENAMES
4360 NEXT LOOP
4370 CLOSE
4380 REM ----- Compare Retention Times -----
4390 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4400 LX=30: LY=328: EST="Please wait.....": GOSUB 20050
4410 FOR LOOP1 = 1 TO NUMSTRTS
4420 IF DPKRT(LOOP1) < 34 THEN RANGE = .625 ELSE RANGE = .5
4430 CHECK = 0: LOOP2 = 1
4440 WHILE CHECK < 1
4450 IF STDT(LOOP2)<(DPKRT(LOOP1)-RANGE) OR
STDT(LOOP2)>(DPKRT(LOOP1)+RANGE) THEN 4490
4460 CHECK = 1
4470 EPRKMS(LOOP1) = ESTOMHYS(LOOP2)
4480 DPKMT(LOOP1)=STDYMEAN(LOOP2)+(STDOSLOPE(LOOP2)*(DPKAREA(LOOP1)-
STDYMEAN(LOOP2)))
4490 LOOP2 = LOOP2 + 1
4500 IF LOOP2 > NUMSTDS AND CHECK < 1 THEN CHECK = 2 ELSE 4530
4510 EPRKMS(LOOP1) = "???"
4520 DPKMT(LOOP1) = 0
4530 WEND
4540 NEXT LOOP1
4550 REM ----- Store Quantitated Data -----
4580 FILEQUANTS = FILES + ".QNT"
4580 OPEN FILEQUANTS AS #1 LEN=27
4600 FIELD #1, 2 AS ENUMSTRTS, 10 AS ESTDTS, 3 AS FLAGS, 12 AS EDUMHYS
4610 LSET ENUMSTRTS = MKIS(NUMSTRTS)
4620 LSET ESTDTS = ESTDATES
4625 LSET FLAGS = "YES"
4630 LSET EDUMHYS = " "
4640 PUT #1,1
4650 FIELD #1, 3 AS ENAMES, 8 AS EPRKMS, 8 AS EPRKMS, 8 AS EPRKMS
4660 FOR LOOP = 1 TO NUMSTRTS
4670 LSET ENAMES = EPRKMS(LOOP)
4680 LSET EPRKMS = MKDS(DPKMT(LOOP))

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4690 LSET EPKAREA = MDS(DPKAREA(LOOP))
4700 LSET EPKAMTS = MDS(DPKAMT(LOOP))
4710 PUT #1, LOOP+1
4720 NEXT LOOP
4730 CLOSE
4740 REM ----- Printout Quantitated Data -----
4750 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4755 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4760 LX=240: LY=328: EST="Check Printer Status": GOSUB 20050
4770 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4780 IF INKEYS = "" THEN 4780
4790 OPEN "LPT1:" AS #3
4800 WIDTH #3,80
4810 PRINT #3, CHR$(27);CHR$(73);CHR$(2): PRINT #3, CHR$(13);CHR$(10)
4820 PRINT #3, TAB(8);"FILE: ";FILES;TAB(34);"INJECTION TIME: ";
INJECTS;TAB(64);EDATES: PRINT #3, CHR$(13);CHR$(10)
4830 PRINT #3, TAB(3);STRINGS(76,205)
4840 PRINT #3, TAB(5);"Peak No.":TAB(20);"Peak Name":TAB(34);
"Retention Time": TAB(55);"Area":TAB(70);"Amount"
4850 PRINT #3, TAB(3);STRINGS(76,196)
4860 FORMAT15 = " "
4870 FORMAT25 = " "
4880 FORMAT35 = " "
4890 FOR LOOP = 1 TO NUMSTRS
4900 PRINT #3, USING FORMAT15;LOOP;
4910 PRINT #3, USING FORMAT25;EPKAMTS(LOOP);
4920 PRINT #3, USING FORMAT35;DPKMT(LOOP);DPKAREA(LOOP);DPKAMT(LOOP)
4930 NEXT LOOP
4940 PRINT #3, TAB(3);STRINGS(76,205)
4950 PRINT #3, TAB(7);"Quantitated data extrapolated from standards recorded
on: ";ESTDATES
4960 IF VAL(MIDS(ESTDATES,4,2)) = VAL(MIDS(EDATES,4,2)) THEN 4990
4970 IF (VAL(MIDS(ESTDATES,1,2))-31) >= (VAL(MIDS(EDATES,1,2))-31) THEN 4990
4980 PRINT #3, CHR$(27);CHR$(69);TAB(16);"WARNING, STANDARD CURVE IS OLDER
THAN ONE MONTH ";CHR$(27);CHR$(73);CHR$(1)
4990 PRINT #3, CHR$(27);CHR$(73);CHR$(1)
5000 PRINT #3, CHR$(12)
5010 CLOSE
5020 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
5025 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
5030 LX=249: LY=328: EST="Another Run (y/n)?:": GOSUB 20050
5033 BEEP
5035 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
5040 QS=INKEYS: IF QS="" THEN 5040
5050 IF QS<>"M" AND QS<>"n" THEN 5060
5055 CALL CLSCR
5057 CHAIN "A:BPLC"
5060 IF QS<>"Y" AND QS<>"y" THEN SOUND 40,10: GOTO 5040
5070 CLEAR
5080 GOTO 220
5099 END
10130 REM ***** Integration Cursor Command Menu *****
10140 REM
10142 LINTS=1
10144 CALL LEVEL(LINTS)
10150 LX=4: LY=328: EST=STRINGS(79,32)
10160 CALL TEXTB(LX,LY,EST)
10170 LX=4: LY=343: EST=STRINGS(79,32)
10180 CALL TEXTB(LX,LY,EST)
10190 LX=27: LY=328: EST="CURSOR COMMANDS: Use L & R arrow
keys -> shift+10X"
10200 CALL TEXTB(LX,LY,EST)
10210 LX=27: LY=343: EST="INTEGRATION COMMANDS: Start End Cancel
Redo Process Quit"
10220 CALL TEXTB(LX,LY,EST)
10222 LINTS=2
10224 CALL LEVEL(LINTS)
10230 RETURN
20000 REM ***** GRAPH-X Subroutine *****
20010 CALL MOVE(LX,LY)
20020 RETURN
20030 CALL DLINE(LX,LY)
20040 RETURN
20050 CALL TEXTB(LX,LY,EST)
20060 RETURN

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MANINTST.SRC

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10 REM PROGRAM NAME = MANINTST.SRC
94 REM
96 REM
200 REM ***** Initilize the Screen and Dimension Variables *****
210 REM
220 DEFINT A,C,B,L,F,V: DEFDBL D,K,R,S: DEFSTR Z,F,I,Q,T,X,Y: DEFSNG B
230 DIM ASTO(5010),BASTO(5010),PSTART(1,25),PREND(1,25),DPKAREA(25),DPKMT(25),
DPKAMT(25),EPKAMTS(25),STDT(20),STDDRM(20),STDMEAN(20),STDSLOE(20),
ESTDAMES(20)
240 PAGE=1: LINTS=1
250 CALL GPAGE(PAGE)
260 CALL LEVEL(LINTS)
270 CALL LEVEL(LINTS)
294 REM
296 REM
400 REM ***** Starting Prompts *****
410 REM
412 CALL CLSCR
413 LX=325: LY=30: EST="Manual Integration of a Stored Chromatogram"
414 CALL TEXTB(LX,LY,EST)
415 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
416 FILES=""
420 LX=20: LY=180: EST="NAME OF FILE FOR INTEGRATION (extension not
permitted) "
421 CALL TEXTB(LX,LY,EST)
422 CALL DISP(PAGE)
423 QS=INKEYS: IF QS="" THEN 423 ELSE CHAR=ASC(QS)
424 IF (CHAR<13 AND CHAR<8) AND (CHAR<32 OR CHAR<126) THEN SOUND 40,10:
GOTO 423
425 IF CHAR=13 THEN 425
426 IF CHAR=8 AND LEN(FILES)<1 THEN SOUND 40,10: GOTO 423
427 IF CHAR=8 AND LEN(FILES)>0 THEN FILES=LEFT$(FILES,(LEN(FILES)-1)):
GOTO 425
428 FILES=FILES+QS
429 LX=518: LY=180: EST=FILES;" "
430 CALL TEXTB(LX,LY,EST)
431 GOTO 423
435 IF MIDS(FILES,2,1) <> "." THEN FILES = "B:" + FILES
440 FOR LOOP = 1 TO LEN(FILES): IF MIDS(FILES,LOOP,1) <> "." THEN NEXT LOOP
ELSE 442
441 GOTO 450
442 LINTS=0
443 CALL LEVEL(LINTS)
444 LX=248: LY=328: EST=" EXTENSION NOT PERMITTED "
445 CALL TEXTB(LX,LY,EST)
446 LINTS=1: BEEP
447 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
448 CALL LEVEL(LINTS)
449 GOTO 412
450 IF LEN(FILES) < 11 THEN 460

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451 LINTS=0
452 CALL LEVEL(LINTS)
453 LX=256: LY=328: EST=" FILE NAME IS TOO LONG "
454 CALL TEXTB(LX,LY,EST)
455 LINTS=1: BEEP
456 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
457 CALL LEVEL(LINTS)
458 GOTO 412
460 LX=20: LY=250: EST="Reading Data, Please Wait....."
462 CALL TEXTB(LX,LY,EST)
465 FILEDATAS = FILES + ".DAT"
470 OPEN FILEDATAS FOR INPUT AS #1
471 INPUT #1,EDATES,INJECTS,NOSAMPLS,MURRTIME,STARTIME
472 FOR LOOP = 1 TO NOSAMPLS STEP 20
473 INPUT #1,B0,A0,B1,A1,B2,A2,B3,A3,B4,A4,B5,A5,B6,A6,B7,A7,B8,A8,B9,
A9,B10,A10,B11,A11,B12,A12,B13,A13,B14,A14,B15,A15,B16,A16,B17,A17,
B18,A18,B19,A19
474 BASTO(LOOP) = B0
475 ASTO(LOOP) = A0
476 BASTO(LOOP+1) = B1
477 ASTO(LOOP+1) = A1
478 BASTO(LOOP+2) = B2
479 ASTO(LOOP+2) = A2
480 BASTO(LOOP+3) = B3
481 ASTO(LOOP+3) = A3
482 BASTO(LOOP+4) = B4
483 ASTO(LOOP+4) = A4
484 BASTO(LOOP+5) = B5
485 ASTO(LOOP+5) = A5
486 BASTO(LOOP+6) = B6
487 ASTO(LOOP+6) = A6
488 BASTO(LOOP+7) = B7
489 ASTO(LOOP+7) = A7
490 BASTO(LOOP+8) = B8
491 ASTO(LOOP+8) = A8
492 BASTO(LOOP+9) = B9
493 ASTO(LOOP+9) = A9
494 BASTO(LOOP+10) = B10
495 ASTO(LOOP+10) = A10
496 BASTO(LOOP+11) = B11
497 ASTO(LOOP+11) = A11
498 BASTO(LOOP+12) = B12
499 ASTO(LOOP+12) = A12
500 BASTO(LOOP+13) = B13
501 ASTO(LOOP+13) = A13
502 BASTO(LOOP+14) = B14
503 ASTO(LOOP+14) = A14
504 BASTO(LOOP+15) = B15
505 ASTO(LOOP+15) = A15
506 BASTO(LOOP+16) = B16
507 ASTO(LOOP+16) = A16
508 BASTO(LOOP+17) = B17
509 ASTO(LOOP+17) = A17
510 BASTO(LOOP+18) = B18
511 ASTO(LOOP+18) = A18
512 BASTO(LOOP+19) = B19
513 ASTO(LOOP+19) = A19
514 NEXT LOOP
515 CLOSE
520 PAGE=0: LINTS=1
530 CALL GPAGE(PAGE)
540 CALL LEVEL(LINTS)
550 CALL CLSCR
594 REM
596 REM
600 REM ***** Draw Running Screen *****
610 REM
620 LX=30: LY=0: GOSUB 20010
630 LX=30: LY=260: GOSUB 20030
632 LX=630: LY=260: GOSUB 20030
634 FOR MARK = 256 TO 0 STEP -10
636 LX=30: LY=MARK: GOSUB 20010
638 LX=25: LY=MARK: GOSUB 20030
640 NEXT MARK
642 FOR MARK = 30 TO 630 STEP 20
644 LX=MARK: LY=260: GOSUB 20010
646 LX=MARK: LY=265: GOSUB 20030
648 NEXT MARK
650 FOR MARK = 30 TO 630 STEP 100
652 LX=MARK: LY=265: GOSUB 20010
654 LX=MARK: LY=270: GOSUB 20030
656 NEXT MARK
658 FOR MARK = 30 TO 630 STEP 200
660 LX=MARK: LY=270: GOSUB 20010
662 LX=MARK: LY=275: GOSUB 20030
664 NEXT MARK
666 LX=21: LY=291: EST="15": GOSUB 20050
668 LX=121: LY=291: EST="20": GOSUB 20050
670 LX=221: LY=291: EST="25": GOSUB 20050
672 LX=321: LY=291: EST="30": GOSUB 20050
674 LX=421: LY=291: EST="35": GOSUB 20050
676 LX=521: LY=291: EST="40": GOSUB 20050
678 LX=621: LY=291: EST="45": GOSUB 20050
680 LX=281: LY=305: EST="TIME (mins)": GOSUB 20050
700 LX=30: LY=343: EST="FILE = " + FILES: GOSUB 20050
710 CALL DISP(PAGE)
796 REM
800 REM ***** Draw Chromatogram *****
810 LX=(CINT((BASTO(1)-15)*20))+30: LY=ASTO(1)
820 CALL PLOT(LX,LY)
830 CALL MOVE(LX,LY)
840 FOR LOOP = 2 TO NOSAMPLS
850 LX=(CINT((BASTO(LOOP)-15)*20))+30: LY=ASTO(LOOP)
860 CALL DLINE(LX,LY)
870 NEXT LOOP
1700 REM ***** Manual Integration *****
1710 REM
1720 LX=18: LY=328: EST=STRINGS(74,32): GOSUB 20050
1730 LX=10: LY=0
1740 CALL MOVE(LX,LY)
1750 LX=10: LY=259
1760 CALL DLINE(LX,LY)
1770 LINTS=2
1780 CALL LEVEL(LINTS)
1890 POSX=10: NUMSTRS=0: NUMENDS=0
1894 PLOTEND = CINT((BASTO(10)-15)*20)+30)
1896 PLOTEND = CINT((BASTO(NOSAMPLS-10)-15)*20)+30)
1900 SAMPOINT = CDBL((NOSAMPLS-20)/(PLOTEND-PLOTEND))
1910 GOSUB 10130
1930 REM ----- Input Control Command -----
1940 INMOVES = INKEYS: IF INMOVES = "" THEN 1940
1942 IF LEN(INMOVES)>1 AND INMOVES=CHR$(0)+"M" THEN MOVE=28: GOTO 1980
1944 IF LEN(INMOVES)>1 AND INMOVES=CHR$(0)+"K" THEN MOVE=29: GOTO 1980
1945 MOVE=ASC(INMOVES)
1950 IF MOVE < 52 THEN 1940
1960 IF MOVE < 55 THEN 1980
1970 IF MOVE=65 AND POSX=29 AND POSX<631 THEN 2070 ELSE SOUND 40,10:
GOTO 1940
1980 REM ----- Move Cursor -----
1990 IF MOVE = 28 THEN POSX2=POSX+1: GOTO 2000
1992 IF MOVE = 54 THEN POSX2=POSX+10: GOTO 2000

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1994 IF MOVE = 29 THEN POSX2=POSX-1: GOTO 2000
1996 IF MOVE = 52 THEN POSX2=POSX-10: GOTO 2000
1998 SOUND 40,10: GOTO 1940
2000 IF POSX2<10 THEN BEEP: POSX2=10
2002 IF POSX2>640 THEN BEEP: POSX2=640
2004 LX=POSX: LY=0
2006 CALL MOVE(LX,LY)
2008 LX=POSX: LY=259
2010 CALL DLINE(LX,LY)
2012 LX=POSX2: LY=259
2014 CALL MOVE(LX,LY)
2016 LX=POSX2: LY=0
2018 CALL DLINE(LX,LY)
2020 POSX=POSX2
2030 GOTO 1940
2040 REM ----- Integration Control Commands -----
2050 REM ----- Peak Start -----
2070 IF MOVE = 83 OR MOVE = 115 THEN SOUND 523.25,.5 ELSE 2230
2080 LOOP = CINT((POSX-35)*SAMPLS)
2090 IF LOOP < 1 THEN LOOP = 1
2100 IF LOOP > NOSAMPLS THEN LOOP = NOSAMPLS
2110 LOWPOINT=0: FLAGS="FIRST"
2120 COMFX = CINT((BSTD(LS(LOOP))-15)*20)+30
2130 WHILE COMFX <= POSX
2140 IF COMFX < POSX THEN 2180
2150 IF FLAGS="FIRST" THEN NUMSTRS=NUMSTRS+1: PKSTART(0,NUMSTRS)=LOOP:
    FLAGS="NO"
    PKSTART(1,NUMSTRS)=LOOP
2170 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2180 LOOP = LOOP + 1
2190 COMFX = CINT((BSTD(LS(LOOP))-15)*20)+30
2200 WEND
2210 LX=POSX: LY=LOWPOINT+1
2211 CALL MOVE(LX,LY)
2212 LX=POSX: LY=LOWPOINT+11
2213 CALL DLINE(LX,LY)
2214 LX=POSX+4: LY=LOWPOINT+11
2215 CALL DLINE(LX,LY)
2216 GOTO 1940
2220 REM ----- Peak End -----
2230 IF MOVE = 69 OR MOVE = 101 THEN SOUND 523.25,.5 ELSE 2390
2240 LOOP = CINT((POSX-25)*SAMPLS)
2250 IF LOOP > NOSAMPLS THEN LOOP = NOSAMPLS
2260 IF LOOP < 1 THEN LOOP = 1
2270 LOWPOINT=0: FLAGS="FIRST"
2280 COMFX = CINT((BSTD(LS(LOOP))-15)*20)+30
2290 WHILE COMFX >= POSX
2300 IF COMFX > POSX THEN 2340
2310 IF FLAGS="FIRST" THEN NUMENDS=NUMENDS+1: PKEND(0,NUMENDS)=LOOP:
    FLAGS="NO"
    PKEND(1,NUMENDS)=LOOP
2330 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2340 LOOP = LOOP - 1
2350 COMFX = CINT((BSTD(LS(LOOP))-15)*20)+30
2360 WEND
2370 LX=POSX: LY=LOWPOINT+1
2371 CALL MOVE(LX,LY)
2372 LX=POSX: LY=LOWPOINT+11
2373 CALL DLINE(LX,LY)
2374 LX=POSX-4: LY=LOWPOINT+11
2375 CALL DLINE(LX,LY)
2376 GOTO 1940
2380 REM ----- Clear Entry -----
2390 IF MOVE < 67 AND MOVE < 99 THEN 2740
2400 CANCEL=0: LOWPOINT=0
2410 IF NUMSTRS < 1 THEN 2570
2420 FOR LOOP = 1 TO NUMSTRS
2430 COMFX = CINT((BSTD(PKSTART(0,LOOP))-15)*20)+30
2440 IF COMFX = POSX THEN CANCEL = LOOP
2450 NEXT LOOP
2460 IF CANCEL < 1 THEN 2570
2470 FOR LOOP = PKSTART(0,CANCEL) TO PKSTART(1,CANCEL)
2480 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2490 NEXT LOOP
2500 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2510 LX=POSX: LY=LOWPOINT+1
2511 CALL MOVE(LX,LY)
2512 LX=POSX: LY=LOWPOINT+11
2513 CALL DLINE(LX,LY)
2514 LX=POSX+4: LY=LOWPOINT+11
2515 CALL DLINE(LX,LY)
2520 FOR LOOP = CANCEL TO NUMSTRS-1
2530 PKSTART(0,LOOP) = PKSTART(0,LOOP+1)
2540 PKSTART(1,LOOP) = PKSTART(1,LOOP+1)
2550 NEXT LOOP
2560 NUMSTRS = NUMSTRS - 1: GOTO 1940
2570 IF NUMSTRS < 1 AND NUMENDS < 1 THEN SOUND 40,10: GOTO 1940
2580 FOR LOOP = 1 TO NUMENDS
2590 COMFX = CINT((BSTD(PKEND(0,LOOP))-15)*20)+30
2600 IF COMFX = POSX THEN CANCEL = LOOP
2610 NEXT LOOP
2620 IF CANCEL < 1 THEN SOUND 40,10: GOTO 1940
2630 FOR LOOP = PKEND(1,CANCEL) TO PKEND(0,CANCEL)
2640 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2650 NEXT LOOP
2660 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2670 LX=POSX: LY=LOWPOINT+1
2671 CALL MOVE(LX,LY)
2672 LX=POSX: LY=LOWPOINT+11
2673 CALL DLINE(LX,LY)
2674 LX=POSX+4: LY=LOWPOINT+11
2675 CALL DLINE(LX,LY)
2680 FOR LOOP = CANCEL TO NUMENDS-1
2690 PKEND(0,LOOP) = PKEND(0,LOOP+1)
2700 PKEND(1,LOOP) = PKEND(1,LOOP+1)
2710 NEXT LOOP
2720 NUMENDS = NUMENDS - 1: GOTO 1940
2730 REM ----- Redo From Start -----
2740 IF MOVE < 82 AND MOVE < 114 THEN 2840
2750 BEEP: LINTS=LINTS-1
2751 CALL LEVEL(LINTS)
2752 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2753 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2754 LX=30: LY=328: EST="Redo From Start.....": GOSUB 20050
2755 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2760 QS = INKEYS: IF QS = "" THEN 2760
2770 IF QS = "y" OR QS = "Y" THEN 2790
2780 GOSUB 10130: GOTO 1940
2790 CALL CLS
2791 LX=30: LY=0: GOSUB 20010
2792 LX=30: LY=260: GOSUB 20030
2793 LX=630: LY=260: GOSUB 20030
2794 FOR MARK = 256 TO 0 STEP -10
2795 LX=30: LY=MARK: GOSUB 20010
2796 LX=25: LY=MARK: GOSUB 20030
2797 NEXT MARK
2798 FOR MARK = 30 TO 630 STEP 20
2799 LX=MARK: LY=260: GOSUB 20010
2800 LX=MARK: LY=265: GOSUB 20030
2801 NEXT MARK
2802 FOR MARK = 30 TO 630 STEP 100
2803 LX=MARK: LY=265: GOSUB 20010
2804 LX=MARK: LY=270: GOSUB 20030
2805 NEXT MARK
2806 FOR MARK = 30 TO 630 STEP 200
2807 LX=MARK: LY=270: GOSUB 20010
2808 LX=MARK: LY=275: GOSUB 20030
2809 NEXT MARK
2810 LX=21: LY=291: EST="15": GOSUB 20050
2811 LX=121: LY=291: EST="20": GOSUB 20050
2812 LX=221: LY=291: EST="25": GOSUB 20050
2813 LX=321: LY=291: EST="30": GOSUB 20050
2814 LX=421: LY=291: EST="35": GOSUB 20050
2815 LX=521: LY=291: EST="40": GOSUB 20050
2816 LX=621: LY=291: EST="45": GOSUB 20050
2817 LX=281: LY=305: EST="TIME (min)": GOSUB 20050
2818 LX=((CINT((BSTD(1)-15)*20))+30): LY=ASTO(1)
2819 CALL PLOT(LX,LY)
2820 CALL MOVE(LX,LY)
2821 FOR LOOP = 2 TO NOSAMPLS
2822 LX=((CINT((BSTD(LOOP)-15)*20))+30): LY=ASTO(LOOP)
2823 CALL DLINE(LX,LY)
2824 NEXT LOOP
2825 GOTO 1700
2830 REM ----- Quit Integration -----
2840 IF MOVE < 81 AND MOVE < 113 THEN 2900
2850 BEEP: LINTS=LINTS-1
2851 CALL LEVEL(LINTS)
2852 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2853 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2854 LX=30: LY=328: EST="Quit.....": GOSUB 20050
2855 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2860 QS = INKEYS: IF QS = "" THEN 2860
2870 IF QS < "y" AND QS < "Y" THEN 2880
2876 CHAIN "A:BPIC"
2880 GOSUB 10130: GOTO 1940
2890 REM ----- Process Data -----
2900 IF MOVE < 80 AND MOVE < 112 THEN SOUND 40,10: GOTO 1940
2910 BEEP: LINTS=LINTS-1
2911 CALL LEVEL(LINTS)
2912 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2913 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2914 LX=30: LY=328: EST="Process.....": GOSUB 20050
2915 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2920 QS = INKEYS: IF QS = "" THEN 2920
2930 IF QS = "y" OR QS = "Y" THEN 2950
2940 GOSUB 10130: GOTO 1940
2950 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2955 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2960 IF NUMSTRS = NUMENDS THEN 3030
2970 BEEP: IF NUMSTRS>NUMENDS THEN 2980
2975 LX=137: LY=328: EST="NUMBER OF PEAK STARTS < NUMBER OF PEAK ENDS":
    GOSUB 20050
2977 GOTO 2990
2980 LX=137: LY=328: EST="NUMBER OF PEAK STARTS > NUMBER OF PEAK ENDS":
    GOSUB 20050
2990 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
3000 IF INKEYS="" THEN 3000
3010 GOSUB 10130: GOTO 1940
3020 REM ----- Bubble Sort -----
3030 REM
3040 CHECK = 1
3050 WHILE CHECK
3060 CHECK = 0
3070 FOR LOOP = 1 TO NUMSTRS-1
3080 IF BSTD(PKSTART(0,LOOP)) <= BSTD(PKSTART(0,LOOP+1)) THEN 3120
3090 SWAP PKSTART(0,LOOP),PKSTART(0,LOOP+1)
3100 SWAP PKSTART(1,LOOP),PKSTART(1,LOOP+1)
3110 CHECK = 1
3120 NEXT LOOP
3130 WEND
3140 CHECK = 1
3150 WHILE CHECK
3160 CHECK = 0
3170 FOR LOOP = 1 TO NUMENDS-1
3180 IF BSTD(PKEND(0,LOOP)) <= BSTD(PKEND(0,LOOP+1)) THEN 3220
3190 SWAP PKEND(0,LOOP),PKEND(0,LOOP+1)
3200 SWAP PKEND(1,LOOP),PKEND(1,LOOP+1)
3210 CHECK = 1
3220 NEXT LOOP
3230 WEND
3240 REM ----- Confirm Order -----
3250 FOR LOOP = 1 TO NUMSTRS
3260 IF PKSTART(0,LOOP) <= PKEND(0,LOOP) THEN 3280
3270 CHECK = CHECK + 1
3280 NEXT LOOP
3290 IF CHECK < 1 THEN 3360
3300 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3310 BEEP: LX=87: LY=328: EST="PEAK STARTS AND PEAK ENDS ARE NOT IN THE
    CORRECT ORDER": GOSUB 20050
3320 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
3330 IF INKEYS="" THEN 3330
3340 GOSUB 10130: GOTO 1940
3350 REM ----- Integration -----
3360 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3364 LX=145: LY=328: EST="Calculating area for peak number": GOSUB 20050
3366 LX=473: LY=328: EST="of"+STR(NUMSTRS): GOSUB 20050
3380 LINTS=2
3381 CALL LEVEL(LINTS)
3382 LX=POSX: LY=0
3383 CALL MOVE(LX,LY)
3384 LX=POSX: LY=259
3385 CALL DLINE(LX,LY)
3386 LINTS=LINTS-1
3387 CALL LEVEL(LINTS)
3390 FOR LOOP1 = 1 TO NUMSTRS
3400 LX=435: LY=328: EST=STR(LOOP1)
3405 CALL TEXTS(LX,LY,EST)
3410 REM ----- Baseline (LSRL) Calculations -----
3420 DSUMX=0: DSUMY=0: DX=0: DXSQ=0: HIGHPOINT=675: FLAGS="NO"
3430 DMSUM=0: DMSUMX=0: DLY=0: DXSQ=0: LOWPOINT=0
3440 FOR LOOP2 = PKSTART(0,LOOP1) TO PKSTART(1,LOOP1)
3450 DSUMX = DSUMX + BSTD(LOOP2)
3460 DSUMY = DSUMY + ASTO(LOOP2)
3470 DX = DX + BSTD(LOOP2)*ASTO(LOOP2)
3480 DXSQ = DXSQ + BSTD(LOOP2)^2
3490 IF ASTO(LOOP2) > LOWPOINT THEN LOWPOINT = ASTO(LOOP2)
3500 NEXT LOOP2
3510 PSTRT = ((CINT((BSTD(PKSTART(0,LOOP1))-15)*20))+30)
3520 LINTS=LINTS-2
3521 CALL LEVEL(LINTS)
3522 LX=PSTRT: LY=LOWPOINT+1
3523 CALL MOVE(LX,LY)
3524 LX=PSTRT: LY=LOWPOINT+11
3525 CALL DLINE(LX,LY)
3526 LX=PSTRT+4: LY=LOWPOINT+11
3527 CALL DLINE(LX,LY)
3528 LOWPOINT = 0
3530 FOR LOOP2 = PKEND(1,LOOP1) TO PKEND(0,LOOP1)
3540 DSUMX = DSUMX + BSTD(LOOP2)
3550 DSUMY = DSUMY + ASTO(LOOP2)
3560

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3570 DXY = DXY + BSTD(LOOP3)*ASTO(LOOP3)
3580 DXSQ = DXSQ + BSTD(LOOP3)*2
3590 IF ASTO(LOOP3) > LOWPOINT THEN LOWPOINT = ASTO(LOOP3)
3600 NEXT LOOP3
3610 PTEND = ((CINT((BSTD(PKEND(0,LOOP1))-15)*20)+30)
3620 LX=PTEND: LY=LOWPOINT+1
3622 CALL MOVE(LX,LY)
3623 LX=PTEND: LY=LOWPOINT+11
3624 CALL DLIN(LX,LY)
3625 LX=PTEND-4: LY=LOWPOINT+11
3626 CALL DLIN(LX,LY)
3627 LINTS=1
3628 CALL LEVEL(LINTS)
3630 N=PKSTART(1,LOOP1)-PKSTART(0,LOOP1)+PKEND(0,LOOP1)-PKEND(1,LOOP1)+2
3640 DXMEAN = DSUMX/N: DYMEAN = DSUMY/N
3650 DLXY=DXY-(DSUMX*DSUMY/N): DLXSQ=DXSQ-((DSUMX*2)/N): SLOPE=DLXY/DLXSQ
3660 REM ----- Calculate Area & Retention Time -----
3670 FOR LOOP4 = PKSTART(0,LOOP1) TO PKEND(0,LOOP1)-1
3680 DBASL = DYMEAN+SLOPE*(BSTD(LOOP4)-DYMEAN)
3700 DBASR = DYMEAN+SLOPE*(BSTD(LOOP4+1)-DYMEAN)
3710 DUPRSID = ABS(ASSTO(LOOP4)-ASTO(LOOP4+1))
3720 DLMSRID = ABS(DBASL-DBASR)
3730 IF ASTO(LOOP4)<ASTO(LOOP4+1) THEN DTEMPY1=ASTO(LOOP4+1) ELSE
3740 DTEMPY1=ASTO(LOOP4)
3750 IF DBASL>DBASR THEN DTEMPY2=DBASR ELSE DTEMPY2=DBASL
3760 DPKAREA(LOOP1)=DPKAREA(LOOP1)+((BSTD(LOOP4+1)-BSTD(LOOP4))
3770 *((.5*DUPRSID)+(.5*DLMSRID)+DTEMPY2-DTEMPY1))
3780 DSTRAT=BSTD(LOOP4): HIGHPOINT=ASTO(LOOP4): FLAG5="END"
3790 IF ASTO(LOOP4)=HIGHPOINT OR FLAG5<>"END" THEN 3800
3800 DENDRT=BSTD(LOOP4-1): FLAG5="NO"
3810 NEXT LOOP4
3820 DPKRT(LOOP1) = DSTRAT*(DENDRT-DSTRAT)/2)
3830 REM ----- Print Retention Times on Screen -----
3840 IS = STR$(DPKRT(LOOP1))
3850 PTX = CINT((DPKRT(LOOP1))-15)*20+27
3860 IF HIGHPOINT < 27 THEN PTY = 27 ELSE PTY = HIGHPOINT
3870 FOR LOOP5 = 2 TO 6
3880 MIDS(IS,LOOP5,1) = "." THEN PTY=PTY-3: GOTO 3920
3890 CHARACTER = VAL(MIDS(IS,LOOP5,1))+1
3900 PTY = PTY - 6
3910 ON CHARACTER GOTO 3923,3932,3938,3947,3960,3966,3977,3988,
3994,4009
3920 LX=PTX+6: LY=PTY: GOSUB 20010
3921 LX=PTX+5: LY=PTY: GOSUB 20030
3922 GOTO 4030
3923 LX=PTX+1: LY=PTY: GOSUB 20010
3924 LX=PTX+5: LY=PTY: GOSUB 20030
3925 LX=PTX+6: LY=PTY+1: GOSUB 20030
3926 LX=PTX+6: LY=PTY+2: GOSUB 20030
3927 LX=PTX+5: LY=PTY+3: GOSUB 20030
3928 LX=PTX+1: LY=PTY+3: GOSUB 20030
3929 LX=PTX: LY=PTY+2: GOSUB 20030
3930 LX=PTX: LY=PTY+1: GOSUB 20030
3931 GOTO 4030
3932 LX=PTX+6: LY=PTY+3: GOSUB 20010
3933 LX=PTX+6: LY=PTY: GOSUB 20030
3934 LX=PTX+5: LY=PTY+1: GOSUB 20030
3935 LX=PTX: LY=PTY+1: GOSUB 20030
3936 LX=PTX+2: LY=PTY+3: GOSUB 20030
3937 GOTO 4030
3938 LX=PTX+6: LY=PTY: GOSUB 20010
3939 LX=PTX+6: LY=PTY+3: GOSUB 20030
3940 LX=PTX+5: LY=PTY+3: GOSUB 20030
3941 LX=PTX+2: LY=PTY: GOSUB 20030
3942 LX=PTX+1: LY=PTY: GOSUB 20030
3943 LX=PTX: LY=PTY+1: GOSUB 20030
3944 LX=PTX: LY=PTY+2: GOSUB 20030
3945 LX=PTX+1: LY=PTY+3: GOSUB 20030
3946 GOTO 4030
3947 LX=PTX+1: LY=PTY+3: GOSUB 20010
3948 LX=PTX+1: LY=PTY+2: GOSUB 20030
3949 LX=PTX: LY=PTY+1: GOSUB 20030
3950 LX=PTX+1: LY=PTY: GOSUB 20030
3951 LX=PTX+2: LY=PTY: GOSUB 20030
3952 LX=PTX+3: LY=PTY+1: GOSUB 20030
3953 LX=PTX+3: LY=PTY+2: GOSUB 20030
3954 LX=PTX+4: LY=PTY: GOSUB 20010
3955 LX=PTX+5: LY=PTY: GOSUB 20030
3956 LX=PTX+6: LY=PTY+1: GOSUB 20030
3957 LX=PTX+6: LY=PTY+2: GOSUB 20030
3958 LX=PTX+5: LY=PTY+3: GOSUB 20030
3959 GOTO 4030
3960 LX=PTX+1: LY=PTY+3: GOSUB 20010
3961 LX=PTX+4: LY=PTY+3: GOSUB 20030
3962 LX=PTX+4: LY=PTY: GOSUB 20030
3963 LX=PTX+6: LY=PTY+1: GOSUB 20010
3964 LX=PTX: LY=PTY+1: GOSUB 20030
3965 GOTO 4030
3966 LX=PTX: LY=PTY: GOSUB 20010
3967 LX=PTX: LY=PTY+3: GOSUB 20030
3968 LX=PTX+2: LY=PTY+3: GOSUB 20030
3969 LX=PTX+2: LY=PTY+1: GOSUB 20030
3970 LX=PTX+3: LY=PTY: GOSUB 20030
3971 LX=PTX+5: LY=PTY: GOSUB 20030
3972 LX=PTX+6: LY=PTY+1: GOSUB 20030
3973 LX=PTX+6: LY=PTY+2: GOSUB 20030
3974 LX=PTX+5: LY=PTY+3: GOSUB 20030
3975 LX=PTX+4: LY=PTY+3: GOSUB 20030
3976 GOTO 4030
3977 LX=PTX+1: LY=PTY: GOSUB 20010
3978 LX=PTX: LY=PTY+1: GOSUB 20030
3979 LX=PTX+2: LY=PTY+3: GOSUB 20030
3980 LX=PTX+5: LY=PTY+3: GOSUB 20030
3981 LX=PTX+6: LY=PTY+2: GOSUB 20030
3982 LX=PTX+6: LY=PTY+1: GOSUB 20030
3983 LX=PTX+5: LY=PTY: GOSUB 20030
3984 LX=PTX+4: LY=PTY: GOSUB 20030
3985 LX=PTX+3: LY=PTY+1: GOSUB 20030
3986 LX=PTX+3: LY=PTY+2: GOSUB 20030
3987 GOTO 4030
3988 LX=PTX: LY=PTY+3: GOSUB 20010
3989 LX=PTX: LY=PTY: GOSUB 20030
3990 LX=PTX+2: LY=PTY: GOSUB 20030
3991 LX=PTX+5: LY=PTY+3: GOSUB 20030
3992 LX=PTX+6: LY=PTY+3: GOSUB 20030
3993 GOTO 4030
3994 LX=PTX+2: LY=PTY+3: GOSUB 20010
3995 LX=PTX+1: LY=PTY+3: GOSUB 20030
3996 LX=PTX: LY=PTY+2: GOSUB 20030
3997 LX=PTX: LY=PTY+1: GOSUB 20030
3998 LX=PTX+1: LY=PTY: GOSUB 20030
3999 LX=PTX+2: LY=PTY: GOSUB 20030
4000 LX=PTX+3: LY=PTY+1: GOSUB 20030
4001 LX=PTX+3: LY=PTY+2: GOSUB 20030
4002 LX=PTX+4: LY=PTY+3: GOSUB 20030
4003 LX=PTX+5: LY=PTY+3: GOSUB 20030
4004 LX=PTX+6: LY=PTY+2: GOSUB 20030
4005 LX=PTX+6: LY=PTY+1: GOSUB 20030
4006 LX=PTX+5: LY=PTY: GOSUB 20030
4007 LX=PTX+4: LY=PTY: GOSUB 20030
4008 GOTO 4030
4009 LX=PTX+6: LY=PTY+2: GOSUB 20010
4010 LX=PTX+4: LY=PTY: GOSUB 20030
4011 LX=PTX+1: LY=PTY: GOSUB 20030
4012 LX=PTX: LY=PTY+1: GOSUB 20030
4013 LX=PTX: LY=PTY+2: GOSUB 20030
4014 LX=PTX+1: LY=PTY+3: GOSUB 20030
4015 LX=PTX+2: LY=PTY+3: GOSUB 20030
4016 LX=PTX+3: LY=PTY+2: GOSUB 20030
4017 LX=PTX+3: LY=PTY+1: GOSUB 20030
4018 NEXT LOOP5
4019 NEXT LOOP1
4020 REM ----- Print Hardcopy of Chromatogram -----
4021 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4022 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4023 LX=619: LY=17: EST=STRINGS(10,32): GOSUB 20050
4024 LX=628: LY=34: EST=STRINGS(8,32): GOSUB 20050
4025 BEEP: LX=96: LY=328: EST="Press <[shift] PrtSc-1> when this message
4026 disappears": GOSUB 20050
4027 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4028 IF INKEY$ = "" THEN 4130
4029 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4030 FOR LOOP = 1 TO 5000: NEXT LOOP
4031 BEEP: LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4032 IF INKEY$ = "" THEN 4170
4033 REM ----- Quantitation -----
4034 REM
4035 REM ----- Read Standard Curve Parameters from A:STANDARDS.DAT -----
4036 LX=30: LY=328: EST=Loading Standard Curve Data.....: GOSUB 20050
4037 OPEN "A:STANDARDS.DAT" AS #2 LEN = 35
4038 GET #2, 2 AS ENUMSTDS, 10 AS EDATIS, 23 AS EDUMYS
4039 GET #2, 1
4040 NUMSTDS = CVT(ENUMSTDS): ESTDATES = EDATIS
4041 FOR LOOP2 = 0 AS ENTS, 0 AS XMEANS, 0 AS YMEANS, 0 AS ESLOPES, 3 AS ENAMES
4042 FIELD LOOP2 = 1 TO NUMSTDS
4043 GET #2, LOOP+1
4044 STDRT(LOOP) = CVD(ENTS)
4045 STDYMEAN(LOOP) = CVD(XMEANS)
4046 STDYMEAN(LOOP) = CVD(YMEANS)
4047 STDSLOPE(LOOP) = CVD(ESLOPES)
4048 ESTONAMS(LOOP) = ENAMES
4049 NEXT LOOP
4050 REM
4051 REM ----- Compare Retention Times -----
4052 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4053 LX=30: LY=328: EST="Please wait.....": GOSUB 20050
4054 FOR LOOP1 = 1 TO NUMSTDS
4055 IF DPKRT(LOOP1) < 34 THEN RANGE = .625 ELSE RANGE = .5
4056 CHECK = 0: LOOP2 = 1
4057 WHILE CHECK < 1
4058 IF STDRT(LOOP2)<(DPKRT(LOOP1)+RANGE) OR
4059 STDRT(LOOP2)>(DPKRT(LOOP1)+RANGE) THEN 4490
4060 CHECK = 1
4061 EPRKAMS(LOOP1) = ESTONAMS(LOOP2)
4062 DPKAMT(LOOP1)=STDYMEAN(LOOP2)+(STDSLOPE(LOOP2)*(DPKAREA(LOOP1)-
4063 STDYMEAN(LOOP2)))
4064 LOOP2 = LOOP2 + 1
4065 IF LOOP2 > NUMSTDS AND CHECK < 1 THEN CHECK = 2 ELSE 4530
4066 EPRKAMS(LOOP1) = "???"
4067 DPKAMT(LOOP1) = 0
4068 WEND
4069 NEXT LOOP1
4070 REM ----- Store Quantitated Data -----
4071 FILEQUANTS = FILES + ".QWT"
4072 OPEN FILEQUANTS AS #1 LEN=27
4073 FIELD #1 2 AS ENUMSTDS, 10 AS ESTDTS, 3 AS FLAGS, 12 AS EDUMYS
4074 LSET ENUMSTDS = MKIS(NUMSTDS)
4075 LSET ESTDTS = ESTDATES
4076 LSET FLAGS = "YES"
4077 LSET EDUMYS = " "
4078 PUT #1, 1
4079 FIELD #1 3 AS ENAMES, 0 AS EPRKTS, 0 AS EPRKAS, 0 AS EPRKAMS
4080 FOR LOOP = 1 TO NUMSTDS
4081 LSET ENAMES = EPRKAMS(LOOP)
4082 LSET EPRKTS = MKDS(DPKRT(LOOP))
4083 LSET EPRKAS = MKDS(DPKAREA(LOOP))
4084 LSET EPRKAMS = MKDS(DPKAMT(LOOP))
4085 PUT #1, LOOP+1
4086 NEXT LOOP
4087 REM ----- Printout Quantitated Data -----
4088 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4089 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4090 LX=240: LY=328: EST="Check Printer Status": GOSUB 20050
4091 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4092 IF INKEY$ = "" THEN 4780
4093 OPEN "LPT1:" AS #3
4094 WIDTH #3,80
4095 PRINT #3, CHR$(27);CHR$(73);CHR$(2): PRINT #3, CHR$(13);CHR$(10)
4096 PRINT #3, TAB(8):"FILE":FILES;TAB(34):"INJECTION TIME":
4097 INJECTS;TAB(64):EDATES:PRINT #3, CHR$(13);CHR$(10)
4098 PRINT #3, TAB(3):STRINGS(76,205)
4099 PRINT #3, TAB(5):"Peak No.":TAB(20):"Peak Name":TAB(34):
4100 "Retention Time": TAB(55):"Area":TAB(70):"Amount"
4101 PRINT #3, TAB(3):STRINGS(76,196)
4102 FORMAT18 = " "
4103 FORMAT28 = " "
4104 FORMAT38 = " "
4105 FOR LOOP = 1 TO NUMSTDS
4106 PRINT #3, USING FORMAT18:LOOP:
4107 PRINT #3, USING FORMAT28:EPRKAMS(LOOP):
4108 PRINT #3, USING FORMAT38:DPKRT(LOOP);DPKAREA(LOOP);DPKAMT(LOOP)
4109 NEXT LOOP
4110 PRINT #3, TAB(3):STRINGS(76,205)
4111 PRINT #3, TAB(7):"Quantitated data extrapolated from standards recorded
4112 on":ESTDATES
4113 IF VAL(MIDS(ESTDATES,4,2)) = VAL(MIDS(EDATES,4,2)) THEN 4990
4114 IF VAL(MIDS(ESTDATES,1,2))-31 >= VAL(MIDS(EDATES,1,2))-31 THEN 4990
4115 PRINT #3, CHR$(27);CHR$(69);TAB(16):"WARNING, STANDARD CURVE IS OLDER
4116 THAN ONE MONTH":CHR$(27);CHR$(70)
4117 PRINT #3, CHR$(27);CHR$(73);CHR$(1)
4118 PRINT #3, CHR$(12)
4119 CLOSE
4120 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4121 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4122 BEEP: LX=222: LY=328: EST="Integrate Another (y/n)?: GOSUB 20050
4123 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 "clear kbrd buffer"
4124 Q8=INKEY$: IF Q8="" THEN 5040
4125 IF Q8<>"n" AND Q8<>"N" THEN 5060
4126 CHAIN "A:BPIC"
4127 IF Q8<>"Y" AND Q8<>"y" THEN SOUND 40,10: GOTO 5040
4128 CLEAR
4129 GOTO 220
4130 END
4131 REM ##### Integration Cursor Command Menu #####
4132 REM
4133 LINTS=1
4134 CALL LEVEL(LINTS)
4135 LX=4: LY=328: EST=STRINGS(79,32)
4136 CALL TEXT9(LX,LY,EST)
4137 LX=4: LY=343: EST=STRINGS(79,32)
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10180 CALL TEXTS(LX,LY,EST)
10190 LX=27: LY=328: EST="CURSOR COMMANDS:          Use L & R arrow
keys -- <shift> = 10X"
10200 CALL TEXTS(LX,LY,EST)
10210 LX=27: LY=343: EST="INTEGRATION COMMANDS:      Start  End  Cancel
Redo  Process  Quit"
10220 CALL TEXTS(LX,LY,EST)
10222 LINTS=2
10224 CALL LEVEL(LINTS)
10230 RETURN
20000 REM ##### GRAPH-X Subroutines #####
20010 CALL MOVE(LX,LY)
20020 RETURN
20030 CALL DLINE(LX,LY)
20040 RETURN
20050 CALL TEXTS(LX,LY,EST)
20060 RETURN

525 OPEN FILEQUANTS AS #1 LEN = 27
530 FIELD #1,2 AS ENUMSTRSS, 10 AS ESTDTS, 3 AS EFLAGS, 12 AS EDUMMYS
535 GET #1,1
540 NUMSTRSS = CVD(ENUMSTRSS)
550 ESTDATES = ESTDTS
555 FLAGS = EFLAGS
556 IF FLAGS <> "YES" THEN CLOSE: KILL FILEQUANTS: FLAGS="NO": GOTO 575
560 FIELD #1, 3 AS ENAMES, 8 AS EPKRTS, 8 AS EPKAREAS, 8 AS EPKAMTS
565 FOR LOOP = 1 TO NUMSTRSS
566   GET #1,LOOP+1
567   EPNAMS(LOOP) = ENAMES
568   DPKRT(LOOP) = CVD(EPKRTS)
569   DPKAREA(LOOP) = CVD(EPKAREAS)
570   DPKAMT(LOOP) = CVD(EPKAMTS)
571 NEXT LOOP
572 CLOSE
575 PAGE=0: LINTS=1
580 CALL GPAGE(PAGE)
585 CALL LEVEL(LINTS)
590 CALL CLRSCL
594 REM
596 REM
600 REM ##### Draw Running Screen #####
610 REM
620 LX=30: LY=0: GOSUB 20010
630 LX=30: LY=260: GOSUB 20030
632 LX=630: LY=260: GOSUB 20030
634 FOR MARK = 256 TO 0 STEP -10
636   LX=30: LY=MARK: GOSUB 20010
638   LX=25: LY=MARK: GOSUB 20030
640 NEXT MARK
642 FOR MARK = 30 TO 630 STEP 20
644   LX=MARK: LY=260: GOSUB 20010
646   LX=MARK: LY=265: GOSUB 20030
648 NEXT MARK
650 FOR MARK = 30 TO 630 STEP 100
652   LX=MARK: LY=265: GOSUB 20010
654   LX=MARK: LY=270: GOSUB 20030
656 NEXT MARK
658 FOR MARK = 30 TO 630 STEP 200
660   LX=MARK: LY=270: GOSUB 20010
662   LX=MARK: LY=275: GOSUB 20030
664 NEXT MARK
666 LX=21: LY=291: EST="15": GOSUB 20050
668 LX=121: LY=291: EST="20": GOSUB 20050
670 LX=221: LY=291: EST="25": GOSUB 20050
672 LX=321: LY=291: EST="30": GOSUB 20050
674 LX=421: LY=291: EST="35": GOSUB 20050
676 LX=521: LY=291: EST="40": GOSUB 20050
678 LX=621: LY=291: EST="45": GOSUB 20050
680 LX=281: LY=305: EST="TIME (min)": GOSUB 20050
700 LX=30: LY=343: EST="FILE = "+FILES: GOSUB 20050
710 CALL DISP(PAGE)
796 REM
800 REM ##### Draw Chromatogram #####
810 LX=(CINT((BSTD(1)-15)*20))+30: LY=ASTO(1)
820 CALL PLOT(LX,LY)
830 CALL MOVE(LX,LY)
840 FOR LOOP = 2 TO NOSAMPLS
850   LX=(CINT((BSTD(LOOP)-15)*20))+30: LY=ASTO(LOOP)
860   CALL DLINE(LX,LY)
870 NEXT LOOP
1700 REM ##### Draw Quantitation Data #####
1710 IF FLAGS = "YES" THEN 1800
1714 LX=4: LY=328: EST=STRINGS(79,32)
1715 CALL TEXTS(LX,LY,EST)
1716 LX=4: LY=343: EST=STRINGS(79,32)
1717 CALL TEXTS(LX,LY,EST)
1718 LINTS=0
1719 CALL LEVEL(LINTS)
1720 LX=168: LY=328: EST="Chromatogram Has Not Been Integrated"
1730 CALL TEXTS(LX,LY,EST)
1734 LINTS=1
1736 CALL LEVEL(LINTS)
1740 LX=195: LY=343: EST="Press any key to continue....."
1750 CALL TEXTS(LX,LY,EST)
1755 IF INKEYS="" THEN 1755
1760 GOTO 4100
1800 REM ----- Print Quantitated Data on Second Graphics Page -----
1810 PAGE=1: LINTS=1
1820 CALL GPAGE(PAGE)
1830 CALL LEVEL(LINTS)
1840 CALL CLRSCL
1850 LX=18: LY=20: EST="FILE: "+FILES
1855 CALL TEXTS(LX,LY,EST)
1860 LX=274: LY=20: EST="INJECTION TIME: "+INJECTS
1865 CALL TEXTS(LX,LY,EST)
1870 LX=611: LY=20: EST=EDATES
1875 CALL TEXTS(LX,LY,EST)
1880 LX=18: LY=34: EST=STRINGS(76,205)
1885 CALL TEXTS(LX,LY,EST)
1890 LX=36: LY=48: EST="Peak No."
1895 CALL TEXTS(LX,LY,EST)
1900 LX=171: LY=48: EST="Peak Name"
1905 CALL TEXTS(LX,LY,EST)
1910 LX=297: LY=48: EST="Retention Time"
1915 CALL TEXTS(LX,LY,EST)
1920 LX=86: LY=68: EST="Area"
1925 CALL TEXTS(LX,LY,EST)
1930 LX=621: LY=48: EST="Amount"
1935 CALL TEXTS(LX,LY,EST)
1940 LX=18: LY=62: EST=STRINGS(76,196)
1945 CALL TEXTS(LX,LY,EST)
1950 FOR LOOP1 = 1 TO NUMSTRSS
1960   LY=62+(LOOP1*14): EST=STRS(LOOP1)
1970   IF LEN(EST)>2 THEN LX=45 ELSE LX=54
1980   CALL TEXTS(LX,LY,EST)
1990   LX=198: LY=62+(LOOP1*14): EST=EPNAMS(LOOP1)
2000   CALL TEXTS(LX,LY,EST)
2010   TEMPS=STRS(DPKRT(LOOP1))
2020   LX=324: LY=62+(LOOP1*14): EST=LEFTS(TEMPS,7)
2030   CALL TEXTS(LX,LY,EST)
2040   TEMPS=STRS(DPKAREA(LOOP1)): CHECK=0: LOOP2=1
2050   WHILE CHECK < 1
2060     TEMPS=MIDS(TEMPS,LOOP2,1)
2070     IF TEMPS<>". " THEN 2110
2080     LX=486-(9*LOOP2)
2090     EST=LEFTS(TEMPS,LOOP2+6)
2100     CHECK=1
2110     LOOP2=LOOP2+1
2120   WEND
2130   LY=62+(LOOP1*14)
2140   CALL TEXTS(LX,LY,EST)
2145   IF DPKAMT(LOOP1)=0 THEN LX=612: EST=" 0.0000": GOTO 2240
2150   TEMPS=STRS(DPKAMT(LOOP1)): CHECK=0: LOOP2=1
2160   WHILE CHECK < 1
2170     TEMPS=MIDS(TEMPS,LOOP2,1)
2180     IF TEMPS<>". " THEN 2220
2190     LX=639-(9*LOOP2)
2200     EST=LEFTS(TEMPS,LOOP2+4)
2210     CHECK=1

```

DISPLAY.SRC

```

10 REM PROGRAM NAME = DISPLAY.SRC
94 REM
96 REM
200 REM ##### Initialize the Screen and Dimension Variables #####
210 REM
220 DEFINT A,C,B,L,P,V: DEFDBL D,K,R,S: DEFSTR E,F,I,Q,T,X,Y: DEFSGN B
230 DIM ASTO(5010),BSTD(5010),PKSTART(1,25),PKEND(1,25),DPKAREA(25),
DPKRT(25),DPKAMT(25),EPNAMS(25),STDR(20),STDMEAN(20),STDTMEAN(20),
STDSLOPE(20),ESTDNAMS(20)
240 PAGE=1: LINTS=1
250 CALL GPAGE(PAGE)
260 CALL LEVEL(LINTS)
294 REM
296 REM
400 REM ##### Starting Prompts #####
410 REM
412 CALL CLRSCL
413 LX=440: LY=30: EST="Display a Stored Chromatogram"
414 CALL TEXTS(LX,LY,EST)
415 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
416 FILES=""
420 LX=20: LY=180: EST="NAME OF FILE TO DISPLAY (extension not permitted) "
421 CALL TEXTS(LX,LY,EST)
422 CALL DISP(PAGE)
423 QS=INKEYS: IF QS="" THEN 423 ELSE CHAR=ASC(QS)
424 IF (CHAR<>13 AND CHAR<>8) AND (CHAR<32 OR CHAR>126) THEN SOUND 40,10:
GOTO 423
425 IF CHAR=13 THEN 435
426 IF CHAR=8 AND LEN(FILES)<1 THEN SOUND 40,10: GOTO 423
427 IF CHAR=8 AND LEN(FILES)>0 THEN FILES=LEFTS(FILES,(LEN(FILES)-1)):
GOTO 429
428 FILES=FILES+QS
429 LX=475: LY=180: EST=FILES+" "
430 CALL TEXTS(LX,LY,EST)
431 GOTO 423
435 IF MIDS(FILES,2,1) <> "." THEN FILES = FILES+FILES
440 FOR LOOP = 1 TO LEN(FILES): IF MIDS(FILES,LOOP,1) <> "." THEN NEXT LOOP
ELSE 442
441 GOTO 450
442 LINTS=0
443 CALL LEVEL(LINTS)
444 LX=248: LY=328: EST=" EXTENSION NOT PERMITTED "
445 CALL TEXTS(LX,LY,EST)
446 LINTS=1: BEEP
447 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
448 CALL LEVEL(LINTS)
449 GOTO 412
450 IF LEN(FILES) < 11 THEN 460
451 LINTS=0
452 CALL LEVEL(LINTS)
453 LX=256: LY=328: EST=" FILE NAME IS TOO LONG "
455 CALL TEXTS(LX,LY,EST)
456 LINTS=1: BEEP
457 FOR LOOP1 = 1 TO 5: FOR LOOP2 = 1 TO 5000: NEXT LOOP2: NEXT LOOP1
458 CALL LEVEL(LINTS)
459 GOTO 412
460 LX=20: LY=250: EST="Reading Data, Please Wait....."
462 CALL TEXTS(LX,LY,EST)
465 FILEDATAS = FILES+".DAT"
470 OPEN FILEDATAS FOR INPUT AS #1
471 INPUT #1,EDATES,INJECTS,NOSAMPLS,KURRTIME,STARTIME
472 FOR LOOP = 1 TO NOSAMPLS STEP 20
473   INPUT #1,B0,A0,B1,A1,B2,A2,B3,A3,B4,A4,B5,A5,B6,A6,B7,A7,B8,A8,B9,
A9,B10,A10,B11,A11,B12,A12,B13,A13,B14,A14,B15,A15,B16,A16,B17,A17,
B18,A18,B19,A19
474   BSTD(LOOP) = B0
475   ASTO(LOOP) = A0
476   BSTD(LOOP+1) = B1
477   ASTO(LOOP+1) = A1
478   BSTD(LOOP+2) = B2
479   ASTO(LOOP+2) = A2
480   BSTD(LOOP+3) = B3
481   ASTO(LOOP+3) = A3
482   BSTD(LOOP+4) = B4
483   ASTO(LOOP+4) = A4
484   BSTD(LOOP+5) = B5
485   ASTO(LOOP+5) = A5
486   BSTD(LOOP+6) = B6
487   ASTO(LOOP+6) = A6
488   BSTD(LOOP+7) = B7
489   ASTO(LOOP+7) = A7
490   BSTD(LOOP+8) = B8
491   ASTO(LOOP+8) = A8
492   BSTD(LOOP+9) = B9
493   ASTO(LOOP+9) = A9
494   BSTD(LOOP+10) = B10
495   ASTO(LOOP+10) = A10
496   BSTD(LOOP+11) = B11
497   ASTO(LOOP+11) = A11
498   BSTD(LOOP+12) = B12
499   ASTO(LOOP+12) = A12
500   BSTD(LOOP+13) = B13
501   ASTO(LOOP+13) = A13
502   BSTD(LOOP+14) = B14
503   ASTO(LOOP+14) = A14
504   BSTD(LOOP+15) = B15
505   ASTO(LOOP+15) = A15
506   BSTD(LOOP+16) = B16
507   ASTO(LOOP+16) = A16
508   BSTD(LOOP+17) = B17
509   ASTO(LOOP+17) = A17
510   BSTD(LOOP+18) = B18
511   ASTO(LOOP+18) = A18
512   BSTD(LOOP+19) = B19
513   ASTO(LOOP+19) = A19
514 NEXT LOOP
515 CLOSE
520 FILEQUANTS=FILES+".QNT"

```



```

2220      LOOP2=LOOP2+1
2230      WEND
2240      LY=62+(LOOP1*14)
2250      CALL TEXTS(LX,LY,EST)
2260      NEXT LOOP1
2270      LX=18: LY=62+((NUMSTRS+1)*14): EST=STRINGS(76,205)
2280      CALL TEXTS(LX,LY,EST)
2290      LX=54: LY=62+((NUMSTRS+2)*14): EST="Quantitated data extrapolated from
standards recorded on: *ESTDATES
2300      CALL TEXTS(LX,LY,EST)
2310      IF VAL(MIDS(ESTDATES,4,2)) = VAL(MIDS(EDATES,4,2)) THEN 2390
2320      IF VAL(MIDS(ESTDATES,1,2))-31 >= (VAL(MIDS(EDATES,1,2))-31) THEN 2390
2330      LINTS=0
2340      CALL LEVEL(LINTS)
2350      LX=140: LY=62+((NUMSTRS+3)*14): EST=" WARNING, STANDARD CURVE IS OLDER
THAN ONE MONTH
2360      CALL TEXTS(LX,LY,EST)
2390      PAGE=0: LINTS=1
2400      CALL LEVEL(LINTS)
2410      CALL GPAGE(PAGE)
2730      REM ----- Print Retention Times on Screen -----
2740      FOR LOOP1 = 1 TO NUMSTRS
2750      LX = CINT((DPKRT(LOOP1)-15)*20)+30
2760      CHECK=0: LOOP2=0
2770      WHILE CHECK<1
2780      LY=LOOP2
2790      CALL GETPT(LX,LY,CHECK)
2800      LOOP2=LOOP2+1
2810      WEND
2815      PTX = CINT((DPKRT(LOOP1)-15)*20)+27
2820      HIGHPOINT = LOOP2-1
2830      IS = STRS(DPKRT(LOOP1))
2840      IF HIGHPOINT < 27 THEN PTY = 27 ELSE PTY = HIGHPOINT
2850      FOR LOOPS = 2 TO 6
2870      IF MIDS(IS,LOOPS,1) = "." THEN PTY=PTY-3: GOTO 3920
2880      CHARACTER = VAL(MIDS(IS,LOOPS,1))+1
2890      PTY = PTY - 6
2900      ON CHARACTER GOTO 3923,3932,3938,3947,3960,3966,3977,
3988,3994,4009
3920      LX=PTX+6: LY=PTY: GOSUB 20010
3921      LX=PTX+5: LY=PTY: GOSUB 20030
3922      GOTO 4030
3923      LX=PTX+1: LY=PTY: GOSUB 20010
3924      LX=PTX+5: LY=PTY: GOSUB 20030
3925      LX=PTX+6: LY=PTY+1: GOSUB 20030
3926      LX=PTX+6: LY=PTY+2: GOSUB 20030
3927      LX=PTX+5: LY=PTY+3: GOSUB 20030
3928      LX=PTX+1: LY=PTY+3: GOSUB 20030
3929      LX=PTX: LY=PTY+2: GOSUB 20030
3930      LX=PTX: LY=PTY+1: GOSUB 20030
3931      GOTO 4030
3932      LX=PTX+6: LY=PTY+3: GOSUB 20010
3933      LX=PTX+6: LY=PTY: GOSUB 20030
3934      LX=PTX+5: LY=PTY+1: GOSUB 20030
3935      LX=PTX: LY=PTY+1: GOSUB 20030
3936      LX=PTX+2: LY=PTY+3: GOSUB 20030
3937      GOTO 4030
3938      LX=PTX+6: LY=PTY: GOSUB 20010
3939      LX=PTX+6: LY=PTY+3: GOSUB 20030
3940      LX=PTX+5: LY=PTY+3: GOSUB 20030
3941      LX=PTX+2: LY=PTY: GOSUB 20030
3942      LX=PTX+1: LY=PTY: GOSUB 20030
3943      LX=PTX: LY=PTY+1: GOSUB 20030
3944      LX=PTX: LY=PTY+2: GOSUB 20030
3945      LX=PTX+1: LY=PTY+3: GOSUB 20030
3946      GOTO 4030
3947      LX=PTX+1: LY=PTY+3: GOSUB 20010
3948      LX=PTX: LY=PTY+2: GOSUB 20030
3949      LX=PTX: LY=PTY+1: GOSUB 20030
3950      LX=PTX+1: LY=PTY: GOSUB 20030
3951      LX=PTX+2: LY=PTY: GOSUB 20030
3952      LX=PTX+3: LY=PTY+1: GOSUB 20030
3953      LX=PTX+3: LY=PTY+2: GOSUB 20030
3954      LX=PTX+4: LY=PTY: GOSUB 20010
3955      LX=PTX+5: LY=PTY: GOSUB 20030
3956      LX=PTX+6: LY=PTY+1: GOSUB 20030
3957      LX=PTX+6: LY=PTY+2: GOSUB 20030
3958      LX=PTX+5: LY=PTY+3: GOSUB 20030
3959      GOTO 4030
3960      LX=PTX+1: LY=PTY+3: GOSUB 20010
3961      LX=PTX+4: LY=PTY+3: GOSUB 20030
3962      LX=PTX+4: LY=PTY: GOSUB 20030
3963      LX=PTX+6: LY=PTY+1: GOSUB 20010
3964      LX=PTX: LY=PTY+1: GOSUB 20030
3965      GOTO 4030
3966      LX=PTX: LY=PTY: GOSUB 20010
3967      LX=PTX: LY=PTY+3: GOSUB 20030
3968      LX=PTX+2: LY=PTY+3: GOSUB 20030
3969      LX=PTX+2: LY=PTY+1: GOSUB 20030
3970      LX=PTX+3: LY=PTY: GOSUB 20030
3971      LX=PTX+5: LY=PTY: GOSUB 20030
3972      LX=PTX+6: LY=PTY+1: GOSUB 20030
3973      LX=PTX+6: LY=PTY+2: GOSUB 20030
3974      LX=PTX+5: LY=PTY+3: GOSUB 20030
3975      LX=PTX+4: LY=PTY+3: GOSUB 20030
3976      GOTO 4030
3977      LX=PTX+1: LY=PTY: GOSUB 20010
3978      LX=PTX: LY=PTY+1: GOSUB 20030
3979      LX=PTX+2: LY=PTY+3: GOSUB 20030
3980      LX=PTX+5: LY=PTY+3: GOSUB 20030
3981      LX=PTX+6: LY=PTY+2: GOSUB 20030
3982      LX=PTX+6: LY=PTY+1: GOSUB 20030
3983      LX=PTX+5: LY=PTY: GOSUB 20030
3984      LX=PTX+4: LY=PTY: GOSUB 20030
3985      LX=PTX+3: LY=PTY+1: GOSUB 20030
3986      LX=PTX+3: LY=PTY+2: GOSUB 20030
3987      GOTO 4030
3988      LX=PTX: LY=PTY+3: GOSUB 20010
3989      LX=PTX: LY=PTY: GOSUB 20030
3990      LX=PTX+2: LY=PTY: GOSUB 20030
3991      LX=PTX+5: LY=PTY+3: GOSUB 20030
3992      LX=PTX+6: LY=PTY+3: GOSUB 20030
3993      GOTO 4030
3994      LX=PTX+2: LY=PTY+3: GOSUB 20010
3995      LX=PTX+1: LY=PTY+3: GOSUB 20030
3996      LX=PTX: LY=PTY+2: GOSUB 20030
3997      LX=PTX: LY=PTY+1: GOSUB 20030
3998      LX=PTX+1: LY=PTY: GOSUB 20030
3999      LX=PTX+2: LY=PTY: GOSUB 20030
4000      LX=PTX+3: LY=PTY+1: GOSUB 20030
4001      LX=PTX+3: LY=PTY+2: GOSUB 20030
4002      LX=PTX+4: LY=PTY+3: GOSUB 20030
4003      LX=PTX+5: LY=PTY+3: GOSUB 20030
4004      LX=PTX+6: LY=PTY+2: GOSUB 20030
4005      LX=PTX+6: LY=PTY+1: GOSUB 20030
4006      LX=PTX+5: LY=PTY: GOSUB 20030
4007      LX=PTX+4: LY=PTY: GOSUB 20030
4008      GOTO 4030
4009      LX=PTX+6: LY=PTY+2: GOSUB 20010
4010      LX=PTX+4: LY=PTY: GOSUB 20030
4011      LX=PTX+1: LY=PTY: GOSUB 20030
4012      LX=PTX: LY=PTY+1: GOSUB 20030
4013      LX=PTX: LY=PTY+2: GOSUB 20030
4014      LX=PTX+1: LY=PTY+3: GOSUB 20030
4015      LX=PTX+2: LY=PTY+3: GOSUB 20030
4016      LX=PTX+3: LY=PTY+2: GOSUB 20030
4017      LX=PTX+3: LY=PTY+1: GOSUB 20030
4030      NEXT LOOP5
4040      NEXT LOOP1
4100      REM ----- Print Hardcopy of Chromatogram -----
4102      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4103      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4104      LX=92: LY=328: EST="Would You Like a Hardcopy of the Chromatogram
(y/n) ?": GOSUB 20050
4105      Q5=INKEY$: IF Q5="" THEN 4105
4106      IF Q5="Y" OR Q5="N" THEN 4180
4107      IF Q5<>"Y" AND Q5<>"N" THEN SOUND 40,10: GOTO 4105
4110      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4112      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4118      BEEP: LX=96: LY=328: EST="Press <[shift] P[rtSc]> when this message
disappears": GOSUB 20050
4120      LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4130      IF INKEY$ = "" THEN 4130
4140      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4150      FOR LOOP = 1 TO 5000: NEXT LOOP
4160      BEEP: LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4170      IF INKEY$ = "" THEN 4170
4180      REM ----- Print Hardcopy of Quantitated Data -----
4190      IF FLAG<>"YES" THEN 5020
4200      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4210      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4220      LX=74: LY=328: EST="Would You Like a Hardcopy of the Quantitated Data
(y/n) ?": GOSUB 20050
4230      Q5=INKEY$: IF Q5="" THEN 4230
4240      IF Q5="Y" OR Q5="N" THEN 4600
4250      IF Q5<>"Y" AND Q5<>"N" THEN SOUND 40,10: GOTO 4230
4260      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4270      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4280      LX=240: LY=328: EST="Check Printer Status": GOSUB 20050
4290      LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4300      IF INKEY$ = "" THEN 4300
4310      OPEN "LPT1:" AS #3
4320      WIDTH #3,80
4330      PRINT #3, CHR$(27);CHR$(73);CHR$(2): PRINT #3, CHR$(13);CHR$(10)
4340      PRINT #3, TAB(8);"FILE: ";FILES;TAB(34);"INJECTION TIME: ";
INJECTS;TAB(64);EDATES: PRINT #3, CHR$(13);CHR$(10)
4350      PRINT #3, TAB(3);STRINGS(76,205)
4360      PRINT #3, TAB(5);"Peak No.:";TAB(20);"Peak Name:";TAB(34);
"Retention Time:";TAB(55);"Area:";TAB(70);"Amount"
4370      PRINT #3, TAB(3);STRINGS(76,196)
4380      FORMAT18 = "A"
4390      FORMAT28 = "X"
4400      FORMAT38 = "###.###"
4410      FOR LOOP = 1 TO NUMSTRS
4420      PRINT #3, USING FORMAT18;LOOP:
4430      PRINT #3, USING FORMAT28;EPKNAME(LOOP);
4440      PRINT #3, USING FORMAT38;DPKRT(LOOP);DPKAREA(LOOP);DPKAMT(LOOP)
4450      NEXT LOOP
4460      PRINT #3, TAB(3);STRINGS(76,205)
4470      PRINT #3, TAB(7);"Quantitated data extrapolated from standards
recorded on: *ESTDATES
4480      IF VAL(MIDS(ESTDATES,4,2)) = VAL(MIDS(EDATES,4,2)) THEN 4510
4490      IF VAL(MIDS(ESTDATES,1,2))-31 >= (VAL(MIDS(EDATES,1,2))-31) THEN 4510
4500      PRINT #3, CHR$(27);CHR$(69);TAB(16);"WARNING, STANDARD CURVE IS OLDER
THAN ONE MONTH !";CHR$(27);CHR$(70)
4510      PRINT #3, CHR$(27);CHR$(73);CHR$(1)
4520      PRINT #3, CHR$(12)
4530      CLOSE
4600      REM ----- View Quantitated Data on the CRT -----
4610      IF FLAG<>"YES" THEN 5020
4620      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4630      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4640      LX=56: LY=328: EST="Would You Like to View the Quantitated Data on the
CRT (y/n) ?": GOSUB 20050
4650      Q5=INKEY$: IF Q5="" THEN 4650
4660      IF Q5="Y" OR Q5="N" THEN 5020
4670      IF Q5<>"Y" AND Q5<>"N" THEN SOUND 40,10: GOTO 4650
4680      PAGE=0: LINTS=1
4690      CALL GPAGE(PAGE)
4700      CALL LEVEL(LINTS)
4710      CALL DISP(PAGE)
4720      LX=4: LY=343: EST=STRINGS(79,32)
4730      CALL TEXTS(LX,LY,EST)
4740      LX=63: LY=343: EST="Press any key to return to chromatogram....."
4750      CALL TEXTS(LX,LY,EST)
4760      DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
4770      IF INKEY$="" THEN 4770
4780      PAGE=0: LINTS=1
4790      CALL GPAGE(PAGE)
4800      CALL LEVEL(LINTS)
4810      CALL DISP(PAGE)
5020      LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
5030      LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
5040      BEEP: LX=240: LY=328: EST="View Another (y/n) ?": GOSUB 20050
5050      DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
5060      Q5=INKEY$: IF Q5="" THEN 5060
5070      IF Q5<>"Y" AND Q5<>"N" THEN 5110
5100      CHAIN "A:RPLC"
5110      IF Q5<>"Y" AND Q5<>"N" THEN SOUND 40,10: GOTO 5060
5120      CLEAR
5130      GOTO 220
9999      END
20000      REM ***** CALIBRAT.X Subroutines *****
20010      CALL MOVE(LX,LY)
20020      RETURN
20030      CALL DLINE(LX,LY)
20040      RETURN
20050      CALL TEXTS(LX,LY,EST)
20060      RETURN

```

CALIBRAT.SRC

```

10      REM PROGRAM NAME = CALIBRAT.SRC
94      REM
96      REM
100      REM ***** Initialise the #255 *****
110      REM
120      OUT &H03,&H92
194      REM
196      REM
200      REM ***** Initialise the Screen and Dimension Variables *****
210      REM
230      DEFINT A,C,E,L,P,V: DEFDBL D,K,X,S: DEFSTR E,F,I,Q,T,X,Y: DEFSGN B
235      FILES="A:STRODS"
240      DIM ASTO(5010),BSTO(5010),PKSTART(1,25),PKEND(1,25),SSTOX(25),
SSTOY(25),SSTOXQ(25),SSTOYQ(25),SSTORT(25),N(25),ESTONAM(25),
DPKAREA(25),DPKRT(25),DPKAMT(25),EPKNAME(25)
250      PAGE=0: LINTS=1
260      CALL GPAGE(PAGE)

```



```

270 CALL LEVEL(LINTS)
280 CALL CLASCR
290 CALL DISP(PAGE)
294 REM
296 REM
300 REM ##### Convert Date Format from American to European #####
310 REM
320 EDATES = DATES: MIDS(EDATES,1,2) = MIDS(DATES,4,2):
MIDS(EDATES,4,2) = MIDS(DATES,1,2)
394 REM
396 REM
400 REM ##### Starting Prompts #####
410 REM
411 IF EREPEATS = "YES" THEN 452
412 LX=40: LY=28: EST="This program is designed for the input of standard
data. It is recommended": GOSUB 20050
413 LX=4: LY=56: EST="that at least five runs are made, so that an accurate
standard curve can be": GOSUB 20050
414 LX=4: LY=84: EST="derived. After each run you will select peak starts
and ends, from which areas": GOSUB 20050
415 LX=4: LY=112: EST="will be calculated. Once this is complete, the
retention times will be ": GOSUB 20050
416 LX=4: LY=140: EST="displayed on line 24 and you will be prompted for the
peak name and the amount.": GOSUB 20050
417 LX=4: LY=168: EST="After each input you will see the values displayed
and asked if they are": GOSUB 20050
418 LX=4: LY=196: EST="correct. If you have made an error, it can be
adjusted at this time.": GOSUB 20050
420 LX=225: LY=328: EST="Do you wish to continue (y/n)?:": GOSUB 20050
421 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
422 QS=INKEY$: IF QS="" THEN 422
423 IF QS="Y" OR QS="y" THEN 452
424 IF QS="N" OR QS="n" THEN CHAIN "A:BPLC" ELSE SOUND 40,10: GOTO 421
452 LX=4: LY=328: EST=STRINGS(79,32)
453 CALL TEXTB(LX,LY,EST)
455 LX=167: LY=328: EST="Reading system parameters, please wait....."
456 CALL TEXTB(LX,LY,EST)
460 PAGE=1: LINTS=1
470 CALL GPAGE(PAGE)
480 CALL LEVEL(LINTS)
490 CALL CLASCR
557 OPEN "A:STNDRDS.TMP" AS #1 LEN=45
558 FIELD #1, 2 AS NUMRUNS, 10 AS ENUMSTDS, 3 AS TODAY, 3 AS FLAGS,
28 AS EDUMMYS
559 GET #1,1
560 IF FLAG#<>"YES" THEN NUMRUNS=1: NUMSTDS=0: GOTO 564
562 NUMRUNS = CUI(ENUMRUNS): NUMRUNS = NUMRUNS+1: NUMSTDS = CUI(ENUMSTDS)
564 CLOSE
570 LX=95: LY=174: EST=" ARE YOU USING AN EXTERNAL TRIGGER TO START
SAMPLING (y/n) ? "
572 CALL TEXTB(LX,LY,EST)
573 CALL DISP(PAGE)
575 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
580 EXTRS = INKEY$: IF EXTRS = "" THEN 580
590 IF EXTRS <> "N" AND EXTRS <> "n" AND EXTRS <> "Y" AND EXTRS <> "y" THEN
SOUND 40,10: GOTO 575
594 REM
596 REM
600 REM ##### Draw Running Screen #####
610 REM
612 PAGE=0: LINTS=1
614 CALL GPAGE(PAGE)
616 CALL LEVEL(LINTS)
618 CALL CLASCR
620 LX=30: LY=0: GOSUB 20010
630 LX=30: LY=260: GOSUB 20030
632 LX=630: LY=260: GOSUB 20030
634 FOR MARK = 256 TO 0 STEP -10
636 LX=30: LY=MARK: GOSUB 20010
638 LX=25: LY=MARK: GOSUB 20030
640 NEXT MARK
642 FOR MARK = 30 TO 630 STEP 20
644 LX=MARK: LY=260: GOSUB 20010
646 LX=MARK: LY=265: GOSUB 20030
648 NEXT MARK
650 FOR MARK = 30 TO 630 STEP 100
652 LX=MARK: LY=265: GOSUB 20010
654 LX=MARK: LY=270: GOSUB 20030
656 NEXT MARK
658 FOR MARK = 30 TO 630 STEP 200
660 LX=MARK: LY=270: GOSUB 20010
662 LX=MARK: LY=275: GOSUB 20030
664 NEXT MARK
666 LX=21: LY=291: EST="15": GOSUB 20050
668 LX=121: LY=291: EST="20": GOSUB 20050
670 LX=221: LY=291: EST="25": GOSUB 20050
672 LX=321: LY=291: EST="30": GOSUB 20050
674 LX=421: LY=291: EST="35": GOSUB 20050
676 LX=521: LY=291: EST="40": GOSUB 20050
678 LX=621: LY=291: EST="45": GOSUB 20050
680 LX=281: LY=305: EST="TIME (mins)": GOSUB 20050
690 LX=619: LY=17: EST=EDATES: GOSUB 20050
700 LX=30: LY=343: EST="FILE = ": GOSUB 20050
710 LX=450: LY=328: EST="Sample Number = ": GOSUB 20050
720 LX=450: LY=343: EST="Run Time = ": GOSUB 20050
730 CALL DISP(PAGE)
796 REM
800 REM ##### Sample Channel 0 -- External Trigger #####
810 REM
820 IF EXTRS = "Y" OR EXTRS = "y" THEN CHANNEL = 0 ELSE GOTO 1100
830 GOSUB 10000
840 LX=30: LY=328: EST="Waiting for External Trigger.....": GOSUB 20050
850 WHILE VALUE < 2500
860 GOSUB 10000
870 WEND
894 REM
896 REM
1100 REM ##### Sample Channel 1 -- Absorbance Data from HPLC #####
1110 REM
1120 CHANNEL=1: NOSAMPLES=0: LASTX=31: LASTY=256: RUNTIME=0: BSTO(0)=.006
1130 LX=30: LY=328: EST=STRINGS(35,32): GOSUB 20050
1140 LX=30: LY=328: EST="Sampling.....": GOSUB 20050
1150 STARTIME = TIMER
1155 LX=30: LY=256
1156 CALL MOVE(LX,LY)
1160 WHILE RUNTIME <= 45
1170 GOSUB 10000
1180 NOSAMPLES = NOSAMPLES + 1
1190 ABVAL = 260 - ((CINT(VALUE/16))+4)
1200 RUNTIME = TIMER
1210 RUNTIME = ((RUNTIME-STARTIME)/60)+15
1215 IF RUNTIME < BSTO(NOSAMPLES-1)+.006 THEN 1200
1220 PLTIME = ((CINT((RUNTIME-15)*20))+30)
1230 LX=PLTIME: LY=ABVAL
1240 CALL DLIN(LX,LY)
1250 BSTO(NOSAMPLES) = RUNTIME: ASTO(NOSAMPLES) = ABVAL
1260 LX=540: LY=343: TEMPS=STRS(RUNTIME): EST=LEFTS(TEMPS,10)
1265 CALL TEXTB(LX,LY,EST)
1270 LX=585: LY=328: EST=STRS(NOSAMPLES)
1275 CALL TEXTB(LX,LY,EST)
1280 LX=628: LY=34: EST=TIME$
1285 CALL TEXTB(LX,LY,EST)
1290 WEND
1294 REM
1296 REM
1700 REM ##### Manual Integration #####
1710 REM
1720 LX=18: LY=328: EST=STRINGS(74,32): GOSUB 20050
1725 LX=628: LY=34: EST=STRINGS(8,32): GOSUB 20050
1730 LX=10: LY=0
1740 CALL MOVE(LX,LY)
1750 LX=10: LY=259
1760 CALL DLIN(LX,LY)
1770 LINTS=2
1780 CALL LEVEL(LINTS)
1890 POSX=10: NUMSTRTS=0: NUMENDS=0
1894 PLTEND = CINT(((BSTO(10)-15)*20)+30)
1896 PLTEND = CINT(((BSTO(NOSAMPLES-10)-15)*20)+30)
1900 SAMPPOINT = CDBL((NOSAMPLES-20)/(PLTEND-PLTEND))
1910 GOSUB 10130
1920 REM ----- Input Control Command -----
1940 INMOVES = INKEY$: IF INMOVES = "" THEN 1940
1942 IF LEN(INMOVES)>1 AND INMOVES=CHR$(0)+"M" THEN MOVE=28: GOTO 1980
1943 IF LEN(INMOVES)>1 AND INMOVES=CHR$(0)+"N" THEN MOVE=29: GOTO 1980
1945 MOVE=ASC(INMOVES)
1950 IF MOVE < 52 THEN 1940
1960 IF MOVE < 55 THEN 1980
1970 IF MOVE>65 AND POSX<29 AND POSX<631 THEN 2070 ELSE SOUND 40,10:
GOTO 1940
1980 REM ----- Move Cursor -----
1990 IF MOVE = 28 THEN POSX2=POSX+1: GOTO 2000
1992 IF MOVE = 54 THEN POSX2=POSX+10: GOTO 2000
1994 IF MOVE = 29 THEN POSX2=POSX-1: GOTO 2000
1996 IF MOVE = 52 THEN POSX2=POSX-10: GOTO 2000
1998 SOUND 40,10: GOTO 1940
2000 IF POSX2<10 THEN BEEP: POSX2=10
2002 IF POSX2>640 THEN BEEP: POSX2=640
2004 LX=POSX: LY=0
2006 CALL MOVE(LX,LY)
2008 LX=POSX: LY=259
2010 CALL DLIN(LX,LY)
2012 LX=POSX2: LY=259
2014 CALL MOVE(LX,LY)
2016 LX=POSX2: LY=0
2018 CALL DLIN(LX,LY)
2020 POSX=POSX2
2030 GOTO 1940
2040 REM ----- Integration Control Commands -----
2050 REM ----- Peak Start -----
2070 IF MOVE = 83 OR MOVE = 115 THEN SOUND 523.25,.5 ELSE 2230
2080 LOOP = CINT((POSX-35)*SAMPPOINT)
2090 IF LOOP < 1 THEN LOOP = 1
2100 IF LOOP > NOSAMPLES THEN LOOP = NOSAMPLES
2110 LOWPOINT=0: FLAGS="FIRST"
2120 COMFX = CINT((BSTO(LOOP)-15)*20)+30
2130 WHILE COMFX <= POSX
2140 IF COMFX < POSX THEN 2150
2150 IF FLAGS="FIRST" THEN NUMSTRTS=NUMSTRTS+1:
PKSTART(0,NUMSTRTS)=LOOP: FLAGS="NO"
2160 PKSTART(1,NUMSTRTS)=LOOP
2170 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2180 LOOP = LOOP + 1
2190 COMFX = CINT((BSTO(LOOP)-15)*20)+30
2200 WEND
2210 LX=POSX: LY=LOWPOINT+1
2211 CALL MOVE(LX,LY)
2212 LX=POSX: LY=LOWPOINT+11
2213 CALL DLIN(LX,LY)
2214 LX=POSX+4: LY=LOWPOINT+11
2215 CALL DLIN(LX,LY)
2216 GOTO 1940
2220 REM ----- Peak End -----
2230 IF MOVE = 69 OR MOVE = 101 THEN SOUND 523.25,.5 ELSE 2390
2240 LOOP = CINT((POSX-25)*SAMPPOINT)
2250 IF LOOP > NOSAMPLES THEN LOOP = NOSAMPLES
2260 IF LOOP < 1 THEN LOOP = 1
2270 LOWPOINT=0: FLAGS="FIRST"
2280 COMFX = CINT((BSTO(LOOP)-15)*20)+30
2290 WHILE COMFX >= POSX
2300 IF COMFX > POSX THEN 2340
2310 IF FLAGS="FIRST" THEN NUMENDS=NUMENDS+1: PKEND(0,NUMENDS)=LOOP:
FLAGS="NO"
2320 PKEND(1,NUMENDS)=LOOP
2330 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2340 LOOP = LOOP - 1
2350 COMFX = CINT((BSTO(LOOP)-15)*20)+30
2360 WEND
2370 LX=POSX: LY=LOWPOINT+1
2371 CALL MOVE(LX,LY)
2372 LX=POSX: LY=LOWPOINT+11
2373 CALL DLIN(LX,LY)
2374 LX=POSX+4: LY=LOWPOINT+11
2375 CALL DLIN(LX,LY)
2376 GOTO 1940
2380 REM ----- Clear Entry -----
2390 IF MOVE <> 67 AND MOVE <> 99 THEN 2740
2400 CANCEL=0: LOWPOINT=0
2410 IF NUMSTRTS < 1 THEN 2570
2420 FOR LOOP = 1 TO NUMSTRTS
2430 COMFX = CINT((BSTO(PKSTART(0,LOOP))-15)*20)+30
2440 IF COMFX = POSX THEN CANCEL = LOOP
2450 NEXT LOOP
2460 IF CANCEL < 1 THEN 2570
2470 FOR LOOP = PKSTART(0,CANCEL) TO PKSTART(1,CANCEL)
2480 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2490 NEXT LOOP
2500 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2510 LX=POSX: LY=LOWPOINT+1
2511 CALL MOVE(LX,LY)
2512 LX=POSX: LY=LOWPOINT+11
2513 CALL DLIN(LX,LY)
2514 LX=POSX+4: LY=LOWPOINT+11
2515 CALL DLIN(LX,LY)
2520 FOR LOOP = CANCEL TO NUMSTRTS-1
2530 PKSTART(0,LOOP) = PKSTART(0,LOOP+1)
2540 PKSTART(1,LOOP) = PKSTART(1,LOOP+1)
2550 NEXT LOOP
2560 NUMSTRTS = NUMSTRTS - 1: GOTO 1940
2570 IF NUMSTRTS < 1 AND NUMENDS < 1 THEN SOUND 40,10: GOTO 1940
2580 FOR LOOP = 1 TO NUMENDS
2590 COMFX = CINT((BSTO(PKEND(0,LOOP))-15)*20)+30
2600 IF COMFX = POSX THEN CANCEL = LOOP
2610 NEXT LOOP
2620 IF CANCEL < 1 THEN SOUND 40,10: GOTO 1940
2630 FOR LOOP = PKEND(1,CANCEL) TO PKEND(0,CANCEL)
2640 IF ASTO(LOOP) > LOWPOINT THEN LOWPOINT = ASTO(LOOP)
2650 NEXT LOOP
2660 SOUND 500,.5: SOUND 32767,1: SOUND 600,.5
2670 LX=POSX: LY=LOWPOINT+1
2671 CALL MOVE(LX,LY)
2672 LX=POSX: LY=LOWPOINT+11

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2673 CALL DLINE(LX,LY)
2674 LX=POSX-4: LY=LOWPOINT+11
2675 CALL DLINE(LX,LY)
2680 FOR LOOP = CANCEL TO NUMENDS-1
2690   PKEND(0,LOOP) = PKEND(0,LOOP+1)
2700   PKEND(1,LOOP) = PKEND(1,LOOP+1)
2710 NEXT LOOP
2720 NUMENDS = NUMENDS - 1: GOTO 1940
2730 REM ----- Redo From Start -----
2740 IF MOVE < 82 AND MOVE < 114 THEN 2840
2750 BEEP: LINTS=1
2751 CALL LEVEL(LINTS)
2752 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2753 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2754 LX=30: LY=328: EST="Redo From Start.....": GOSUB 20050
2755 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2760 QS = INKEYS: IF QS = "" THEN 2760
2770 IF QS = "Y" OR QS = "Y" THEN 2790
2780 GOSUB 10130: GOTO 1940
2790 CALL CLASCAR
2791 LX=30: LY=0: GOSUB 20010
2792 LX=30: LY=260: GOSUB 20030
2793 LX=630: LY=260: GOSUB 20030
2794 FOR MARK = 256 TO 0 STEP -10
2795   LX=30: LY=MARK: GOSUB 20010
2796   LX=25: LY=MARK: GOSUB 20030
2797 NEXT MARK
2798 FOR MARK = 30 TO 630 STEP 20
2799   LX=MARK: LY=260: GOSUB 20010
2800   LX=MARK: LY=265: GOSUB 20030
2801 NEXT MARK
2802 FOR MARK = 30 TO 630 STEP 100
2803   LX=MARK: LY=265: GOSUB 20010
2804   LX=MARK: LY=270: GOSUB 20030
2805 NEXT MARK
2806 FOR MARK = 30 TO 630 STEP 200
2807   LX=MARK: LY=270: GOSUB 20010
2808   LX=MARK: LY=275: GOSUB 20030
2809 NEXT MARK
2810 LX=21: LY=291: EST="15": GOSUB 20050
2811 LX=121: LY=291: EST="20": GOSUB 20050
2812 LX=221: LY=291: EST="25": GOSUB 20050
2813 LX=321: LY=291: EST="30": GOSUB 20050
2814 LX=421: LY=291: EST="35": GOSUB 20050
2815 LX=521: LY=291: EST="40": GOSUB 20050
2816 LX=621: LY=291: EST="45": GOSUB 20050
2817 LX=281: LY=305: EST="TIME (mins)": GOSUB 20050
2818 LX=(CINT((BSTO(1)-15)*20))+30: LY=ASTO(1)
2819 CALL MOVE(LX,LY)
2820 CALL MOVE(LX,LY)
2821 FOR LOOP = 2 TO NOSAMPLES
2822   LX=(CINT((BSTO(LOOP)-15)*20))+30: LY=ASTO(LOOP)
2823   CALL DLINE(LX,LY)
2824 NEXT LOOP
2825 GOTO 1700
2830 REM ----- Quit Integration -----
2840 IF MOVE < 81 AND MOVE < 113 THEN 2900
2850 BEEP: LINTS=1
2851 CALL LEVEL(LINTS)
2852 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2853 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2854 LX=30: LY=328: EST="Quit.....": GOSUB 20050
2855 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2860 QS = INKEYS: IF QS = "" THEN 2860
2870 IF QS < "Y" AND QS < "Y" THEN 2880
2872 CALL CLASCAR
2876 CHAIN "A:EPIC"
2880 GOSUB 10130: GOTO 1940
2890 REM ----- Process Data -----
2900 IF MOVE < 80 AND MOVE < 112 THEN SOUND 40,10: GOTO 1940
2910 BEEP: LINTS=1
2911 CALL LEVEL(LINTS)
2912 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2913 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2914 LX=30: LY=328: EST="Process.....": GOSUB 20050
2915 LX=227: LY=343: EST="CONFIRM SELECTION (y/n)": GOSUB 20050
2920 QS = INKEYS: IF QS = "" THEN 2920
2930 IF QS = "Y" OR QS = "Y" THEN 2950
2940 GOSUB 10130: GOTO 1940
2950 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
2955 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
2960 IF NUMSTRTS = NUMENDS THEN 3030
2970 BEEP: IF NUMSTRTS=NUMENDS THEN 2980
2975 LX=137: LY=328: EST="NUMBER OF PEAK STARTS < NUMBER OF PEAK ENDS": GOSUB 20050
2977 GOTO 2990
2980 LX=137: LY=328: EST="NUMBER OF PEAK STARTS > NUMBER OF PEAK ENDS": GOSUB 20050
2990 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
3000 IF INKEYS="" THEN 3000
3010 GOSUB 10130: GOTO 1940
3020 REM ----- Bubble Sort -----
3030 REM
3040 CHECK = 1
3050 WHILE CHECK
3060   CHECK = 0
3070   FOR LOOP = 1 TO NUMSTRTS-1
3080     IF BSTO(PKSTART(0,LOOP)) <= BSTO(PKSTART(0,LOOP+1)) THEN 3120
3090     SWAP PKSTART(0,LOOP),PKSTART(0,LOOP+1)
3100     SWAP PKSTART(1,LOOP),PKSTART(1,LOOP+1)
3110     CHECK = 1
3120   NEXT LOOP
3130 NEWB
3140 CHECK = 1
3150 WHILE CHECK
3160   CHECK = 0
3170   FOR LOOP = 1 TO NUMENDS-1
3180     IF BSTO(PKEND(0,LOOP)) <= BSTO(PKEND(0,LOOP+1)) THEN 3220
3190     SWAP PKEND(0,LOOP),PKEND(0,LOOP+1)
3200     SWAP PKEND(1,LOOP),PKEND(1,LOOP+1)
3210     CHECK = 1
3220   NEXT LOOP
3230 NEWB
3240 REM ----- Confirm Order -----
3250 FOR LOOP = 1 TO NUMSTRTS
3260   IF PKSTART(0,LOOP) <= PKEND(0,LOOP) THEN 3280
3270   CHECK = CHECK + 1
3280 NEXT LOOP
3290 IF CHECK < 1 THEN 3360
3300 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3310 BEEP: LX=7: LY=328: EST="PEAK STARTS AND PEAK ENDS ARE NOT IN THE CORRECT ORDER": GOSUB 20050
3320 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
3330 IF INKEYS="" THEN 3330
3340 GOSUB 10130: GOTO 1940
3350 REM ----- Integration -----
3360 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
3364 LX=145: LY=328: EST="Calculating area for peak number": GOSUB 20050
3366 LX=473: LY=328: EST="of "+STR$(NUMSTRTS): GOSUB 20050
3380 LINTS=2
3381 CALL LEVEL(LINTS)
3382 LX=POSX: LY=0
3383 CALL MOVE(LX,LY)
3384 LX=POSX: LY=259
3385 CALL DLINE(LX,LY)
3386 LINTS=1
3387 CALL LEVEL(LINTS)
3390 FOR LOOP1 = 1 TO NUMSTRTS
3400   LX=435: LY=328: EST=STR$(LOOP1)
3405   CALL TEXTB(LX,LY,EST)
3410   REM ----- Baseline (LSRL) Calculations -----
3420   DSUMX=0: DSUMY=0: DXY=0: DXSQ=0: HIGHPOINT=675: FLAGS="NO"
3420   DXMEAN=0: DYMEAN=0: DLWMSQ=0: DLXSQ=0: LOWPOINT=0
3430   PKSTART(0,LOOP1) TO PKSTART(1,LOOP1)
3440   DSUMX = DSUMX + BSTO(LOOP2)
3450   DSUMY = DSUMY + ASTO(LOOP2)
3460   DXY = DXY + BSTO(LOOP2)*ASTO(LOOP2)
3470   DXSQ = DXSQ + BSTO(LOOP2)^2
3480   IF ASTO(LOOP2) > LOWPOINT THEN LOWPOINT = ASTO(LOOP2)
3490 NEXT LOOP2
3500   PTSTRT = ((CINT((BSTO(PKSTART(0,LOOP1))-15)*20))+30)
3510   LINTS=2
3520   CALL LEVEL(LINTS)
3522   LX=PTSTRT: LY=LOWPOINT+1
3524   CALL MOVE(LX,LY)
3525   LX=PTSTRT: LY=LOWPOINT+11
3526   CALL DLINE(LX,LY)
3527   LX=PTSTRT+4: LY=LOWPOINT+11
3528   CALL DLINE(LX,LY)
3530   LOWPOINT = 0
3540   FOR LOOP3 = PKEND(1,LOOP1) TO PKEND(0,LOOP1)
3550     DSUMX = DSUMX + BSTO(LOOP3)
3560     DSUMY = DSUMY + ASTO(LOOP3)
3570     DXY = DXY + BSTO(LOOP3)*ASTO(LOOP3)
3580     DXSQ = DXSQ + BSTO(LOOP3)^2
3590     IF ASTO(LOOP3) > LOWPOINT THEN LOWPOINT = ASTO(LOOP3)
3600 NEXT LOOP3
3610   PTEND = ((CINT((BSTO(PKEND(0,LOOP1))-15)*20))+30)
3620   LX=PTEND: LY=LOWPOINT+1
3622   CALL MOVE(LX,LY)
3623   LX=PTEND: LY=LOWPOINT+11
3624   CALL DLINE(LX,LY)
3625   LX=PTEND+4: LY=LOWPOINT+11
3626   CALL DLINE(LX,LY)
3627   LINTS=1
3628   CALL LEVEL(LINTS)
3630   N=PKSTART(1,LOOP1)-PKSTART(0,LOOP1)+PKEND(0,LOOP1)-PKEND(1,LOOP1)+2
3640   DXMEAN = DSUMX/N: DYMEAN = DSUMY/N
3650   DLWMSQ = (DSUMX*DSUMX)/(N): DLXSQ = (DSUMY*DSUMY)/(N): SLOPE=DLX/DLXSQ
3660   REM ----- Calculate Area & Retention Time -----
3670   FOR LOOP4 = PKSTART(0,LOOP1) TO PKEND(0,LOOP1)-1
3680     DBASL = DYMEAN+SLOPE*(BSTO(LOOP4)-DXMEAN)
3690     DBASR = DYMEAN+SLOPE*(BSTO(LOOP4+1)-DXMEAN)
3700     DUFASID = ABS(ASTO(LOOP4)-ASTO(LOOP4+1))
3710     DLWMSID = ABS(DBASL-DBASR)
3720     IF ASTO(LOOP4)<ASTO(LOOP4+1) THEN DTEMPY1=ASTO(LOOP4+1)
3730     ELSE DTEMPY1=ASTO(LOOP4)
3740     IF DBASL>DBASR THEN DTEMPY2=DBASR ELSE DTEMPY2=DBASL
3750     DFKAREA(LOOP1)=OPKAREA(LOOP1)+((BSTO(LOOP4+1)-BSTO(LOOP4))*((.5*DUFASID)+(.5*DLWMSID)+DTEMPY2-DTEMPY1))
3760     IF ASTO(LOOP4)>=HIGHPOINT THEN 3780
3770     DSTART=ASTO(LOOP4): HIGHPOINT=ASTO(LOOP4): FLAGS="END"
3780     IF ASTO(LOOP4)=HIGHPOINT OR FLAGS<>"END" THEN 3800
3790     DENDRT=BSTO(LOOP4-1): FLAGS="NO"
3800 NEXT LOOP4
3810   DPKRT(LOOP1) = DSTART+((DENDRT-DSTART)/2)
3820   REM ----- Print Retention Times on Screen -----
3830   IS = STR$(DPKRT(LOOP1))
3840   PTX = CINT((DPKRT(LOOP1)-15)*20)+27
3850   IF HIGHPOINT < 27 THEN PTY = 27 ELSE PTY = HIGHPOINT
3860   FOR LOOPS = 2 TO 6
3870     IF MIDS(IS,LOOPS,1) = "." THEN PTY=PTY-3: GOTO 3920
3880     CHARACTER = VAL(MIDS(IS,LOOPS,1))+1
3890     PTY = PTY - 6
3900   ON CHARACTER GOTO 3923,3932,3938,3947,3960,3966,3977,3981,3984,4009
3910   LX=PTX+6: LY=PTY: GOSUB 20010
3920   LX=PTX+5: LY=PTY: GOSUB 20030
3922   GOTO 4030
3923   LX=PTX+1: LY=PTY: GOSUB 20010
3924   LX=PTX+5: LY=PTY: GOSUB 20030
3925   LX=PTX+6: LY=PTY+1: GOSUB 20030
3926   LX=PTX+6: LY=PTY+2: GOSUB 20030
3927   LX=PTX+5: LY=PTY+3: GOSUB 20030
3928   LX=PTX+1: LY=PTY+3: GOSUB 20030
3929   LX=PTX+1: LY=PTY+2: GOSUB 20030
3930   LX=PTX: LY=PTY+1: GOSUB 20030
3931   GOTO 4030
3932   LX=PTX+6: LY=PTY+3: GOSUB 20010
3933   LX=PTX+6: LY=PTY: GOSUB 20030
3934   LX=PTX+5: LY=PTY+1: GOSUB 20030
3935   LX=PTX: LY=PTY+1: GOSUB 20030
3936   LX=PTX+2: LY=PTY+3: GOSUB 20030
3937   GOTO 4030
3938   LX=PTX+6: LY=PTY: GOSUB 20010
3939   LX=PTX+6: LY=PTY+3: GOSUB 20030
3940   LX=PTX+5: LY=PTY+3: GOSUB 20030
3941   LX=PTX+2: LY=PTY: GOSUB 20030
3942   LX=PTX+1: LY=PTY: GOSUB 20030
3943   LX=PTX: LY=PTY+1: GOSUB 20030
3944   LX=PTX: LY=PTY+2: GOSUB 20030
3945   LX=PTX+1: LY=PTY+3: GOSUB 20030
3946   GOTO 4030
3947   LX=PTX+1: LY=PTY+3: GOSUB 20010
3948   LX=PTX: LY=PTY+2: GOSUB 20030
3949   LX=PTX: LY=PTY+1: GOSUB 20030
3950   LX=PTX+1: LY=PTY: GOSUB 20030
3951   LX=PTX+2: LY=PTY: GOSUB 20030
3952   LX=PTX+3: LY=PTY+1: GOSUB 20030
3953   LX=PTX+3: LY=PTY+2: GOSUB 20030
3954   LX=PTX+4: LY=PTY: GOSUB 20010
3955   LX=PTX+5: LY=PTY: GOSUB 20030
3956   LX=PTX+6: LY=PTY+1: GOSUB 20030
3957   LX=PTX+6: LY=PTY+2: GOSUB 20030
3958   LX=PTX+5: LY=PTY+3: GOSUB 20030
3959   GOTO 4030
3960   LX=PTX+1: LY=PTY+3: GOSUB 20010
3961   LX=PTX+4: LY=PTY+3: GOSUB 20030
3962   LX=PTX+4: LY=PTY: GOSUB 20030
3963   LX=PTX+6: LY=PTY+1: GOSUB 20010
3964   LX=PTX: LY=PTY+1: GOSUB 20030
3965   GOTO 4030
3966   LX=PTX: LY=PTY: GOSUB 20010
3967   LX=PTX: LY=PTY+3: GOSUB 20030
3968   LX=PTX+2: LY=PTY+3: GOSUB 20030
3969   LX=PTX+2: LY=PTY+1: GOSUB 20030
3970   LX=PTX+3: LY=PTY: GOSUB 20030
3971   LX=PTX+5: LY=PTY: GOSUB 20030
3972   LX=PTX+6: LY=PTY+1: GOSUB 20030
3973   LX=PTX+6: LY=PTY+2: GOSUB 20030
3974   LX=PTX+5: LY=PTY+3: GOSUB 20030
3975   LX=PTX+4: LY=PTY+3: GOSUB 20030

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3976 GOTO 4030
3977 LX=PTX+1: LY=PTY: GOSUB 20010
3978 LX=PTX: LY=PTY+1: GOSUB 20030
3979 LX=PTX+2: LY=PTY+3: GOSUB 20030
3980 LX=PTX+5: LY=PTY+3: GOSUB 20030
3981 LX=PTX+6: LY=PTY+2: GOSUB 20030
3982 LX=PTX+6: LY=PTY+1: GOSUB 20030
3983 LX=PTX+5: LY=PTY: GOSUB 20030
3984 LX=PTX+4: LY=PTY: GOSUB 20030
3985 LX=PTX+3: LY=PTY+1: GOSUB 20030
3986 LX=PTX+3: LY=PTY+2: GOSUB 20030
3987 GOTO 4030
3988 LX=PTX: LY=PTY+3: GOSUB 20010
3989 LX=PTX: LY=PTY: GOSUB 20030
3990 LX=PTX+2: LY=PTY: GOSUB 20030
3991 LX=PTX+5: LY=PTY+3: GOSUB 20030
3992 LX=PTX+6: LY=PTY+3: GOSUB 20030
3993 GOTO 4030
3994 LX=PTX+2: LY=PTY+3: GOSUB 20010
3995 LX=PTX+1: LY=PTY+3: GOSUB 20030
3996 LX=PTX: LY=PTY+2: GOSUB 20030
3997 LX=PTX: LY=PTY+1: GOSUB 20030
3998 LX=PTX+1: LY=PTY: GOSUB 20030
3999 LX=PTX+2: LY=PTY: GOSUB 20030
4000 LX=PTX+3: LY=PTY+1: GOSUB 20030
4001 LX=PTX+3: LY=PTY+2: GOSUB 20030
4002 LX=PTX+4: LY=PTY+3: GOSUB 20030
4003 LX=PTX+5: LY=PTY+3: GOSUB 20030
4004 LX=PTX+6: LY=PTY+2: GOSUB 20030
4005 LX=PTX+6: LY=PTY+1: GOSUB 20030
4006 LX=PTX+5: LY=PTY: GOSUB 20030
4007 LX=PTX+4: LY=PTY: GOSUB 20030
4008 GOTO 4030
4009 LX=PTX+6: LY=PTY+2: GOSUB 20010
4010 LX=PTX+4: LY=PTY: GOSUB 20030
4011 LX=PTX+1: LY=PTY: GOSUB 20030
4012 LX=PTX: LY=PTY+1: GOSUB 20030
4013 LX=PTX: LY=PTY+2: GOSUB 20030
4014 LX=PTX+1: LY=PTY+3: GOSUB 20030
4015 LX=PTX+2: LY=PTY+3: GOSUB 20030
4016 LX=PTX+3: LY=PTY+2: GOSUB 20030
4017 LX=PTX+3: LY=PTY+1: GOSUB 20030
4018 NEXT LOOP5
4019 NEXT LOOP1
4020 LX=4: LY=328: EST=STRINGS(79,32)
4021 CALL TEXTB(LX,LY,EST)
4022 LX=4: LY=343: EST=STRINGS(79,32)
4023 CALL TEXTB(LX,LY,EST)
4024 LX=92: LY=328: EST="Would you like a hardcopy of the chromatogram (y/n) ?"
4025 BEEP
4026 CALL TEXTB(LX,LY,EST)
4027 QHRCDFYS=INKEY$: IF QHRCDFYS="" THEN 4064
4028 IF QHRCDFYS="M" OR QHRCDFYS="N" THEN 4180
4029 IF QHRCDFYS<>"Y" AND QHRCDFYS<>"N" THEN SOUND 40,10: GOTO 4064
4030 LX=4: LY=328: EST=STRINGS(79,32)
4031 CALL TEXTB(LX,LY,EST)
4032 REM ----- Print Hardcopy of Chromatogram -----
4033 LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4034 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4035 LX=618: LY=17: EST=STRINGS(10,32): GOSUB 20050
4036 LX=628: LY=34: EST=STRINGS(8,32): GOSUB 20050
4037 BEEP: LX=96: LY=328: EST="Press <shift> PrtSc-> when this message disappears": GOSUB 20050
4038 LX=195: LY=343: EST="Press any key to continue.....": GOSUB 20050
4039 IF INKEY$="" THEN 4130
4040 LX=4: LY=343: EST=STRINGS(79,32): GOSUB 20050
4041 FOR LOOP = 1 TO 5000: NEXT LOOP
4042 BEEP: LX=4: LY=328: EST=STRINGS(79,32): GOSUB 20050
4043 IF INKEY$="" THEN 4170
4044 REM ----- Enter Standard Values -----
4045 REM
4046 LX=4: LY=328: EST=STRINGS(79,32)
4047 CALL TEXTB(LX,LY,EST)
4048 LX=4: LY=343: EST=STRINGS(79,32)
4049 CALL TEXTB(LX,LY,EST)
4050 FOR LOOP = 1 TO NUMSTRTS
4051 LX=30: LY=328: EST="PK NO.: "+STR$(LOOP)
4052 CALL TEXTB(LX,LY,EST)
4053 TEMP1=STR$(DPKMT$(LOOP))
4054 TEMP2=LEFT$(TEMP1,7)
4055 LX=167: LY=328: EST="PK RT: "+TEMP2
4056 CALL TEXTB(LX,LY,EST)
4057 LX=240: LY=343: EST="Enter Peak Name: "+EPKNAME$(LOOP)=""
4058 CALL TEXTB(LX,LY,EST)
4059 Q$=INKEY$: IF Q$="" THEN 4231
4060 CHAR=ASC(Q$)
4061 IF (CHAR<13 AND CHAR<8) AND (CHAR<65 OR CHAR<90) AND (CHAR<97 OR CHAR<122) THEN SOUND 40,10: GOTO 4231
4062 IF CHAR=13 THEN 4241
4063 IF CHAR=8 AND LEN(EPKNAME$(LOOP))<1 THEN SOUND 40,10: GOTO 4231
4064 IF CHAR=8 AND LEN(EPKNAME$(LOOP))>0 THEN EPKNAME$(LOOP)=LEFT$(EPKNAME$(LOOP), (LEN(EPKNAME$(LOOP))-1)): GOTO 4238
4065 EPKNAME$(LOOP)=EPKNAME$(LOOP)+Q$
4066 LX=394: LY=343: EST=EPKNAME$(LOOP)+" "
4067 CALL TEXTB(LX,LY,EST)
4068 GOTO 4231
4069 LX=381: LY=328: EST="PK NAME: "+EPKNAME$(LOOP)
4070 CALL TEXTB(LX,LY,EST)
4071 LX=4: LY=343: EST=STRINGS(79,32)
4072 CALL TEXTB(LX,LY,EST)
4073 LX=209: LY=343: EST="Enter Peak Amount:"
4074 CALL TEXTB(LX,LY,EST)
4075 TEMP$=""
4076 Q$=INKEY$: IF Q$="" THEN 4248
4077 CHAR=ASC(Q$)
4078 IF (CHAR<13 AND CHAR<8 AND CHAR<46) AND (CHAR<48 OR CHAR<57) THEN SOUND 40,10: GOTO 4248
4079 IF CHAR=13 THEN 4258
4080 IF CHAR=8 AND LEN(TEMP$)<1 THEN SOUND 40,10: GOTO 4248
4081 IF CHAR=8 AND LEN(TEMP$)>0 THEN TEMP$=LEFT$(TEMP$, (LEN(TEMP$)-1)): GOTO 4255
4082 TEMP$=TEMP$+Q$
4083 LX=381: LY=343: EST=TEMP$+" "
4084 CALL TEXTB(LX,LY,EST)
4085 GOTO 4248
4086 LX=486: LY=328: EST="PK AMT: "+LEFT$(TEMP$,8)
4087 CALL TEXTB(LX,LY,EST)
4088 DPKMT$(LOOP)=VAL(TEMP$)
4089 LX=4: LY=343: EST=STRINGS(79,32)
4090 CALL TEXTB(LX,LY,EST)
4091 BEEP: LX=186: LY=343: EST="Are these values correct (y/n) ?"
4092 CALL TEXTB(LX,LY,EST)
4093 DEF SEG=0: POKE 1050, FREE(1052): DEF SEG: POKE 106,0 'clear keyboard
4094 Q$=INKEY$: IF Q$="" THEN 4290
4095 IF Q$="Y" OR Q$="y" THEN 4330
4096 IF Q$<>"M" AND Q$<>"N" THEN SOUND 40,10: GOTO 4280
4097 LX=4: LY=328: EST=STRINGS(79,32)
4098 CALL TEXTB(LX,LY,EST)
4099 LX=4: LY=343: EST=STRINGS(79,32)
4100 CALL TEXTB(LX,LY,EST)
4101 GOTO 4030
4102 GOTO 4210
4103 LX=4: LY=328: EST=STRINGS(79,32)
4104 CALL TEXTB(LX,LY,EST)
4105 LX=4: LY=343: EST=STRINGS(79,32)
4106 CALL TEXTB(LX,LY,EST)
4107 LX=4: LY=343: EST=STRINGS(79,32)
4108 CALL TEXTB(LX,LY,EST)
4109 BEEP: LX=240: LY=328: EST="Check Printer Status"
4110 CALL TEXTB(LX,LY,EST)
4111 LX=195: LY=343: EST="Press any key to continue....."
4112 CALL TEXTB(LX,LY,EST)
4113 IF INKEY$="" THEN 4780
4114 OPEN "LPT1:" AS #3
4115 MIOFF #3,80
4116 PRINT #3, CHR$(27);CHR$(73);CHR$(2): PRINT #3, CHR$(13);CHR$(10)
4117 PRINT #3, TAB(8);"FILE: "+FILES(TAB(34));"RUN NUMBER: "+NUMRUNS: TAB(64);EDATES: PRINT #3, CHR$(13);CHR$(10)
4118 PRINT #3, TAB(3);STRINGS(76,205)
4119 PRINT #3, TAB(5);"Peak No.":TAB(20);"Peak Name":TAB(34);"Retention Time":TAB(55);"Area":TAB(70);"Amount"
4120 PRINT #3, TAB(3);STRINGS(76,196)
4121 FORMAT1$=" "
4122 FORMAT2$=" "
4123 FORMAT3$=" "
4124 FOR LOOP = 1 TO NUMSTRTS
4125 PRINT #3, USING FORMAT1$:LOOP;
4126 PRINT #3, USING FORMAT2$:EPKNAME$(LOOP);
4127 PRINT #3, USING FORMAT3$:DPKMT$(LOOP);DPKAREA$(LOOP);DPKAMT$(LOOP)
4128 NEXT LOOP
4129 PRINT #3, TAB(3);STRINGS(76,205)
4130 PRINT #3, CHR$(27);CHR$(73);CHR$(1)
4131 PRINT #3, CHR$(12)
4132 CLOSE
4133 LX=4: LY=328: EST=STRINGS(79,32)
4134 CALL TEXTB(LX,LY,EST)

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5024 LX=4: LY=343: EST=STRINGS(79,32)
5026 CALL TEXTS(LX,LY,EST)
5028 LX=168: LY=328: EST="Repeat"
5030 CALL TEXTS(LX,LY,EST)
5032 LX=267: LY=328: EST="Process"
5034 CALL TEXTS(LX,LY,EST)
5036 LX=375: LY=328: EST="Stop"
5038 CALL TEXTS(LX,LY,EST)
5040 LX=465: LY=328: EST="Quit"
5042 CALL TEXTS(LX,LY,EST)
5044 LX=231: LY=343: EST="Enter your choice....."
5046 CALL TEXTS(LX,LY,EST)
5050 DEF SEG=0: POKE 1050,PEEK(1052): DEF SEG: POKE 106,0 'clear kbrd buffer
5060 IMOVES=INKEY$: IF IMOVES="" THEN 5060 ELSE MOVE=ASC(IMOVES)
5070 IF (MOVE>79 AND MOVE<84) OR (MOVE=111 AND MOVE<116) THEN 5085
5080 SOUND 40,10: GOTO 5050
5085 REM ----- Repeat Run -----
5090 IF MOVE<>82 AND MOVE<>114 THEN 5130
5095 CLOSE
5100 CLEAR
5110 EREPEATS="YES"
5120 PAGE=0: GOTO 230
5130 REM ----- Stop -----
5140 IF MOVE<>83 AND MOVE<>115 THEN 5180
5150 CLOSE
5160 CLEAR
5162 CALL CLRSCL
5170 CHAIN "A:RPLC"
5180 REM ----- Quit -----
5190 IF MOVE<>81 AND MOVE<>113 THEN 5250
5200 CLOSE
5202 LX=4: LY=328: EST=STRINGS(79,32)
5203 CALL TEXTS(LX,LY,EST)
5204 LX=4: LY=343: EST=STRINGS(79,32)
5205 CALL TEXTS(LX,LY,EST)
5206 LINTS=0
5207 CALL LEVEL(LINTS)
5208 BEEP: LX=182: LY=343: EST=" Enter Y to confirm loss of data "
5209 CALL TEXTS(LX,LY,EST)
5210 LINTS=1
5211 CALL LEVEL(LINTS)
5212 QS=INKEY$: IF QS="" THEN 5210
5213 IF QS<>"Y" AND QS<>"y" THEN 5020
5214 CALL CLRSCL
5220 KILL "A:STNDRDS.TMP"
5230 CLEAR
5240 CHAIN "A:RPLC"
5250 REM ----- Process -----
5260 IF MOVE<>80 AND MOVE<>112 THEN SOUND 40,10: GOTO 5050
5261 LX=4: LY=328: EST=STRINGS(79,32)
5262 CALL TEXTS(LX,LY,EST)
5263 LX=4: LY=343: EST=STRINGS(79,32)
5264 CALL TEXTS(LX,LY,EST)
5265 LX=10: LY=328: EST="Processing data, please wait....."
5266 CALL TEXTS(LX,LY,EST)
5270 CLOSE: KILL "A:STNDRDS.DAT"
5280 OPEN "A:STNDRDS.TMP" AS #1 LEN=45
5290 FIELD #1, 2 AS ENUMSTDS, 2 AS ENUMSTDS, 10 AS ESTDATES, 3 AS FLAGS,
28 AS EDUMMYS
5300 GET #1,1
5310 NUMSTDS=CVI(ENUMSTDS): NUMSTDS=CVI(ENUMSTDS)
5330 FIELD #1, 8 AS XS, 8 AS YS, 8 AS XYS, 8 AS XSQS, 8 AS ERTS, 2 AS ENS,
3 AS ENAMS
5340 OPEN "A:STNDRDS.DAT" AS #2 LEN=35
5350 FIELD #2, 2 AS ENUMSTDS, 10 AS ESTDATES, 23 AS EDUMMYS
5360 LSET ENUMSTDS = MKIS(NUMSTDS)
5370 LSET ESTDATES = TODAY$
5380 LSET EDUMMYS = " "
5390 PUT #2,1
5400 FIELD #2, 8 AS ESTORTS, 8 AS XMEANS, 8 AS YMEANS, 8 AS ESLOPES,
3 AS ESTONAMS
5410 FOR LOOP = 1 TO NUMSTDS
5420 GET #1,LOOP+1
5430 SUMX = CVD(XS)
5440 SUMY = CVD(YS)
5450 SUMXY = CVD(XYS)
5460 SUMXSQ = CVD(XSQS)
5470 SUMYT = CVD(ERTS)
5480 N = CVI(ENS)
5490 STORT = SUMRT/N
5500 STDMEAN = SUMX/N
5510 STDYMEAN = SUMY/N
5520 SUMXLXY = SUMXY-(SUMX*SUMY/N)
5530 SUMXLXQ = SUMXSQ-((SUMX*2)/N)
5540 STDSLOPE = SUMXLXY/SUMXLXQ
5550 LSET ESTORTS = MKDS(STORT)
5560 LSET XMEANS = MKDS(STDMEAN)
5570 LSET YMEANS = MKDS(STDYMEAN)
5580 LSET ESLOPES = MKDS(STDSLOPE)
5590 LSET ESTONAMS = ENAMS
5600 PUT #2,LOOP+1
5610 NEXT LOOP
5620 CLOSE
5630 LX=4: LY=328: EST=STRINGS(79,32)
5632 CALL TEXTS(LX,LY,EST)
5634 LX=4: LY=343: EST=STRINGS(79,32)
5636 CALL TEXTS(LX,LY,EST)
5638 LX=186: LY=328: EST="Standards File Has Been Updated."
5640 CALL TEXTS(LX,LY,EST)
5642 BEEP: LX=168: LY=343: EST="Press any key to return to menu....."
5644 CALL TEXTS(LX,LY,EST)
5660 IF INKEY$="" THEN 5660
5670 KILL "A:STNDRDS.TMP"
5680 CLEAR
5690 CHAIN "A:RPLC"
9999 END

10000 REM ##### Sampling Subroutine for Selected Channel (=CHANNEL) #####
10010 REM
10020 OUT &H702,2
10030 OUT &H702,CHANNEL*810+3
10040 VALUE=(INP(&H701)AND &BF)*256+INP(&H700)
10050 RETURN
10060 REM
10070 REM
10130 REM ##### Integration Cursor Command Menu #####
10140 REM
10142 LINTS=1
10144 CALL LEVEL(LINTS)
10150 LX=4: LY=328: EST=STRINGS(79,32)
10160 CALL TEXTS(LX,LY,EST)
10170 LX=4: LY=343: EST=STRINGS(79,32)
10180 CALL TEXTS(LX,LY,EST)
10190 LX=27: LY=328: EST="CURSOR COMMANDS: Use L & R arrow
keys - <shift> = 10X"
10200 CALL TEXTS(LX,LY,EST)
10210 LX=27: LY=343: EST="INTEGRATION COMMANDS: Start End Cancel
Redo Process Quit"
10220 CALL TEXTS(LX,LY,EST)
10222 LINTS=2
10224 CALL LEVEL(LINTS)
10230 RETURN

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